

**Generation of dual T cell receptor (TCR) T cells
by TCR gene transfer
for adoptive T cell therapy**

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Summary

The *in vitro* generation of T cells with a defined antigen specificity by T cell receptor (TCR) gene transfer is an established method to create cells for immunotherapy. However, one major challenge of this strategy is to achieve sufficiently high expression levels of the therapeutic TCR. As T cells which already express an endogenous TCR are equipped with additional TCR α and TCR β chains, there is a competition between therapeutic and endogenous TCR for the invariant TCR components (CD3 and TCR ζ) and cell surface transport. In addition, mixed pairs of endogenous and exogenous TCR chains can be formed. Before this work was started, it was not known which TCR α/β combinations are really present on the cell surface after TCR gene transfer. Therefore, we established models, where we transferred TCR genes into murine and human T cells expressing defined endogenous TCR. After gene transfer, both TCR could be analyzed by staining with antibodies and MHC-multimers. We found that some TCR α/β combinations have the capability to replace other TCR on the cell surface, which led to a complete conversion of antigen specificity in one model. Based on these findings we proposed the concept of “strong” (well expressed) and “weak” (poorly expressed) TCR.

In addition, we found that a mouse TCR is able to replace both “weak” and “strong” human TCR on human cells. In parallel to this result, it was reported that the constant (C)-regions of mouse TCR were responsible for the improved expression of murine TCR on human cells. Based on these findings, a strategy to improve the expression of human TCR was developed by exchanging the human C-regions by their murine counterparts (murinization). We systematically compared murinization to other published optimization strategies that had yielded higher TCR expression levels (including additional cystein bonds and codon-optimization). Using different TCR, we found that especially when optimizing “weak” TCR a striking improvement of expression and function could be achieved. Best results were obtained when combining codon-optimization, which leads to enhanced protein levels, and murinization, which enhances the preferential pairing and the stability of the transferred TCR α/β combination.

However, a potential problem of murinization of human TCR is the likely immunogenicity of these hybrid constructs, due to the complete mouse gene segments. Therefore, we identified the specific parts of the mouse C-regions of the TCR α and TCR β chains which are needed to increase TCR expression and function of TCR gene modified cells. The identification was performed with a series of hybrid constructs, which included different domains of the murine C-regions. Strikingly, in the TCR β C-region one amino acid exchange from an acidic glutamic acid (human) to a basic lysine (mouse) at position 18 was found to be most important. Four additional “murine” amino acids further improved the TCR expression. Within the TCR α C-region, a domain of four amino acids was found to be sufficient for the enhanced expression. To show a broad applicability, minimally murinized variants

(nine amino acids from the mouse sequence) of different TCR were tested in primary human T cells. Cells modified with minimally murinized TCR had a higher TCR expression level and released significant more interferon- γ after coculture with cells presenting the antigen compared to cells modified with the wild type TCR. For TCR gene therapy the utilization of minimally instead of completely murinized C-regions will reduce the amount of foreign sequences and thus the risk of immunogenicity of the therapeutic TCR.

Keywords: T cell receptor (TCR), TCR replacement, TCR optimization, murinization

Zusammenfassung

Die Herstellung von T-Zellen mit definierten Spezifitäten durch den Transfer von T-Zellrezeptor (TCR, *engl.* T cell receptor) Genen ist eine etablierte Methode, um Zellen für eine Immuntherapie bereitzustellen. Eine besondere Herausforderung ist jedoch, ein ausreichend hohes Expressionsniveau des therapeutischen TCR zu erreichen. Da T-Zellen, die bereits einen endogenen TCR exprimieren, mit zusätzlichen TCR α und TCR β Ketten ausgestattet werden, entsteht eine Konkurrenzsituation zwischen dem therapeutischen und dem endogenen TCR um die Komponenten des TCR Komplexes (CD3 und TCR ζ) und um den Transport an die Zelloberfläche. Zusätzlich können gemischte TCR entstehen, die aus einer endogenen und einer exogenen TCR Kette bestehen. Bevor diese Arbeit begonnen wurde war nicht bekannt, welche TCR α/β Kombinationen nach dem Transfer von TCR Genen auf der Zelloberfläche exprimiert werden. Daher haben wir Modelle etabliert, in denen TCR Gene in Maus und humane T-Zellen mit definierten endogenen TCR transferiert wurden. Nach dem Gentransfer konnten beide TCR mithilfe von Antikörpern und MHC-Multimeren angefärbt werden. Diese Modelle haben gezeigt, dass bestimmte TCR α/β Kombinationen andere TCR von der Zelloberfläche verdrängen können; dies führte in einem Fall zu einer vollständigen Umkehr der Antigenpezifität. Aufgrund dieser Ergebnisse haben wir das Konzept von „starken“ (gut exprimierten) und „schwachen“ (schlecht exprimierten) TCR vorgeschlagen.

Zusätzlich wurde die Verdrängung von „schwachen“ und „starken“ humanen TCR durch Maus TCR auf humanen Zellen beobachtet. Parallel zu diesem Ergebnis wurde berichtet, dass die konstanten (C, *engl.* constant) Regionen von Maus TCR für die erhöhte Expression dieser TCR auf humanen Zellen verantwortlich sind. Aufgrund dieser Ergebnisse wurde eine Strategie zur Verbesserung der Expression humaner TCR entwickelt, die auf dem Austausch humaner TCR C-Regionen durch die von Maus TCR basiert (Murinisierung). Wir haben die Murinisierung systematisch mit anderen veröffentlichten Optimierungsstrategien (zusätzliche Disulfidbindung, Codon-Optimierung), die zu einem erhöhten TCR Expressionsniveau geführt hatten, verglichen. Die Ergebnisse mit verschiedenen TCR zeigten, dass, hauptsächlich bei der Optimierung „schwacher“ TCR, eine starke Verbesserung der Expression und der Funktion erreicht werden konnte. Die besten Ergebnisse wurden erzielt, wenn eine Kombination aus Codon-Optimierung, die zu einer Erhöhung der Proteinmenge führt, und Murinisierung, die die bevorzugte Dimerisierung und die Stabilität der transferierten TCR α/β Kombination erhöht, angewendet wurde.

Ein mögliches Problem der Murinisierung könnte die Immunogenität der hybriden Konstrukte aufgrund der kompletten Maus Gensegmente sein. Deshalb haben wir jene Bereiche der Maus C-Regionen identifiziert, die für die erhöhte Expression und die verbesserte Funktion der Gen-modifizierten Zellen verantwortlich sind. Die Identifizierung wurde mit einer Reihe von hybriden

Konstrukten, die unterschiedliche Domänen der Maus C-Regionen enthielten, durchgeführt. In der TCR β Kette wurde ein Austausch von Glutaminsäure zu dem basischen Lysin an Position 18 als wichtigster Unterschied zwischen humaner und Maus Sequenz identifiziert. Die TCR Expression konnte mit vier zusätzlichen Aminosäuren aus der Maus Sequenz weiter erhöht werden. In der C-Region der TCR α Kette wurde eine Domäne aus vier Aminosäuren gefunden, die ausreichend für eine Verbesserung der Expression war. Um eine breite Anwendbarkeit zu zeigen, wurden minimal murinisierte Varianten (neun Aminosäuren der Maus Sequenz) von verschiedenen TCR in primären humanen T-Zellen getestet. T-Zellen, die mit minimal murinisierten TCR modifiziert wurden, zeigten ein höheres TCR Expressionsniveau und setzten mehr Interferon- γ nach Kultivierung mit Antigen präsentierenden Zellen frei als T-Zellen, die mit Wildtyp TCR modifiziert wurden. Für die TCR Gentherapie bedeutet die Verwendung minimal murinisierter anstelle von komplett murinisierten C-Regionen eine Verminderung des Risikos, dass der therapeutische TCR immunogen wirkt.

Schlagworte: T-Zell Rezeptor (TCR), TCR Verdrängung, TCR Optimierung, Murinisierung

1 Introduction

1.1 T cell receptor (TCR)

1.1.1 The TCR complex

T cells play a crucial role in the adaptive immune response. The specificity of a T cell is defined by its T cell receptor (TCR). Most TCR are built up of a variable ligand binding TCR α / β heterodimer, which is stabilized by the invariant dimers CD3 $\gamma\epsilon$ (CD = cluster of differentiation), CD3 $\delta\epsilon$, and $\zeta\zeta$ (Figure 1a) (Kuhns *et al.*, 2006). A small fraction of T cells expresses TCR with TCR γ and TCR δ chains instead of TCR α and TCR β chains. The invariant chains are responsible for signaling. CD3 γ , CD3 δ , and CD3 ϵ consist of an immunoglobulin like extracellular domain, a transmembrane region, and a cytoplasmic C-terminal part with an immunoreceptor tyrosine-based activation motif (ITAM). ITAM contain two tyrosines, which are phosphorylated during signal transduction (Kane *et al.*, 2000). TCR ζ chains have only very small extracellular parts (9 amino acids), but larger cytoplasmic domains than CD3 molecules consisting of three ITAM. The two TCR ζ chains are linked via a disulfide bond. Transmembrane regions of TCR molecules have most probably an α -helical structure (Campbell *et al.*, 1994). In the transmembrane domains of each invariant chain one acidic residue is located. These acidic residues form polar interactions with basic residues in the TCR α and TCR β transmembrane regions (Call and Wucherpfennig, 2007). The two acidic residues of CD3 $\delta\epsilon$ dimers interact with a lysine (K) of TCR α chains (amino acid position is shown in Figure 2a), CD3 $\gamma\epsilon$ with a lysine of TCR β chains (Figure 2b), and $\zeta\zeta$ with an arginine (R) of TCR α chains (Figure 2a) (Call *et al.*, 2002). In this model six acidic residues in the invariant chains interact with only three basic residues in the transmembrane regions of TCR α and TCR β chains. Therefore, also TCR complexes with two TCR α / β heterodimers had been proposed to balance the charges (Fernandez-Miguel *et al.*, 1999). However, most results support a model with only one TCR α / β dimer and a specific interaction of two acidic and one basic residue resulting in three-transmembrane-helix bundles (Call *et al.*, 2002). The three basic residues within the transmembrane region of TCR α and TCR β are conserved across different species and also in all TCR variants ($\alpha\beta$, $\gamma\delta$, and pre-TCR). Extracellular contact sides between the three signaling dimers and the TCR α and TCR β chains are still under debate (Kuhns *et al.*, 2006; Call and Wucherpfennig, 2007). Soluble extracellular domains of CD3 and TCR α or TCR β do not interact with each other (Sun *et al.*, 2004), which indicates that the extracellular interactions are weak and that the transmembrane interactions are needed to enable their formation.

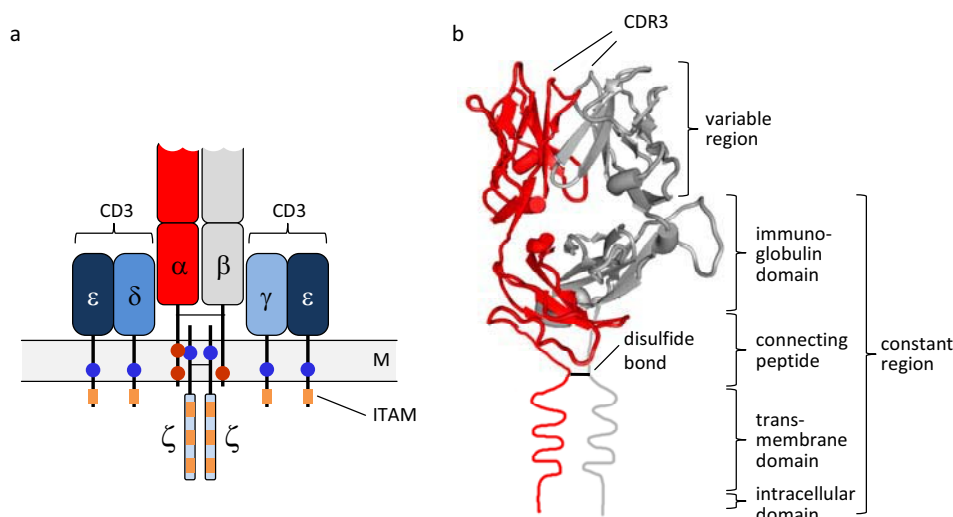


Figure 1: Structure of a TCR. (a) Modified from (Murphy *et al.*, 2008). The TCR complex consists of two variable chains (α and β) and the invariant dimers CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$ and $\zeta\zeta$. Basic residues within the membrane (M) region are shown as red dots and acidic residues as blue dots. Immunoreceptor tyrosine-based activation motifs (ITAM) are marked in orange. (b) Structure of TCR α/β . Variable regions and the immunoglobulin domains of the C-regions are taken from the crystallography structure of a TCR specific for an influenza hemagglutinin peptide (Hennecke and Wiley, 2002). The non-crystallized rest of the C-regions is drawn schematically. TCR α chain is shown in red, TCR β chain in gray.

1.1.2 TCR α and TCR β chains

The first molecular definition of TCR α and TCR β chains was in 1984, when differences in the gene profile between T and B cells were analyzed (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984). TCR α and TCR β chains are glycoproteins linked via a disulfide bond located in the extracellular part (Kuhns *et al.*, 2006). Each chain can be subdivided into a N-terminal variable and a C-terminal constant (C) region (Figure 1b). The variable regions are located extracellularly and include the antigen binding region. They have an immunoglobulin like structure, which is characterized by a pack of β sheets stabilized by an intramolecular disulfide bond (Murphy *et al.*, 2008). Loops connecting the β sheets, which are directed to the top, are responsible for binding the antigen. Each TCR chain has three of these loop regions called complementarity determining regions (CDR). They have large diversity with CDR3 being most variable. The parts between the CDR regions are called framework regions (Figure 2). Although different variable regions differ in their sequence, their overall structures are very similar. The C-regions are divided into an extracellular immunoglobulin like domain, which follows the variable region, a connecting peptide, in which the cysteine forming the disulfide bond is located, a transmembrane domain, which probably has an α -helical structure (Campbell *et al.*, 1994), and a short intracellular domain at the C-terminus (Figure 1b). In TCR α chains the immunoglobulin domain has an untypical structure as half of the domain consists of loosely packed strands and the intramolecular disulfide bond connects a β strand with an α helix.

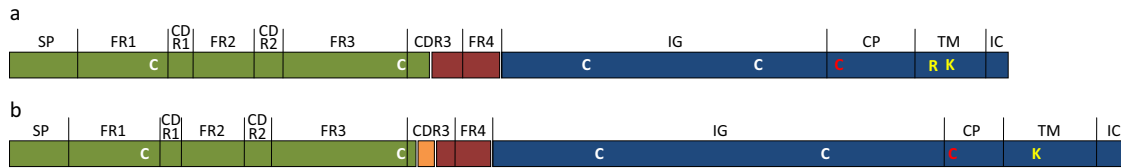


Figure 2: Schematic drawing of the composition of TCR α and TCR β chains. SP: signal peptide, FR: framework region, CDR: complementarity determining region, IG: immunoglobulin like domain of the C-region, CP: connecting peptide, TM: transmembrane domain, IC: intracellular domain. Parts built up by the V segments are green, by the D segment are orange, by the J segment are red, and by the C segment are blue (see Figure 3). Cysteines forming intramolecular disulfide bonds are white, cysteines for the intermolecular disulfide bond are in red, and residues in the transmembrane region with interactions to the invariant TCR chains are yellow.

1.1.3 Organization and rearrangement of TCR α and TCR β loci

The human TCR α locus, which also includes the TCR δ locus, is located on chromosome 14, the TCR β locus on chromosome 7. The variable region of a TCR α chain can be built up by 46 different V α (variable) segments, each preceded by a leader sequence, and 58 J α (joining) segments (Figure 3a). The variable region of a TCR β chain can be formed from 54 V β , 14 J β , and 2 D β (diversity) segments (Rowen *et al.*, 1996) (Figure 3b). There is only one gene segment for C α , but two different for C β . The V, D, and J gene segments are rearranged during T cell development in the thymus (Kragel, 2009) by the enzyme complex V(D)J recombinase, which contains the two components RAG-1 and RAG-2 (RAG: recombination-activating gene) (Fugmann *et al.*, 2000). The recombinase recognizes 12 bp and 23 bp long recombination signal sequences, which flank the TCR gene segments. In addition, nucleotides can be added or deleted at the junction between the segments. The rearrangement of different segments and the addition or deletion of nucleotides results in the extremely high diversity of CDR3 regions. The C-regions are spliced to the variable regions after transcription.

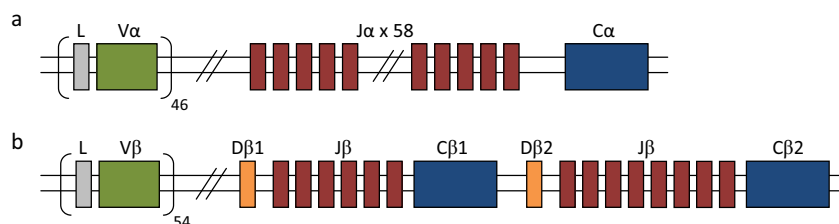


Figure 3: Schematic drawing of the human TCR α and TCR β loci. Modified from (Murphy *et al.*, 2008). (a) The human TCR α gene locus consists of 46 V α (variable), 58 J α (joining) and one C α (constant) segment. (b) The human TCR β gene locus consists of 54 V β , 2D β (diversity) 14 J β and two C β segments. In front of each V segment a leader sequence (L) is located.

1.1.4 Interaction with the major histocompatibility complex (MHC)

TCR recognize peptides bound to the highly polymorphic major histocompatibility complex (MHC) molecules (Zinkernagel and Doherty, 1979). In humans MHC genes are called human leukocyte

antigen (HLA) genes (Dausset, 1958). There are two classes of MHC molecules: class I MHC are recognized by CD8⁺ and class II MHC by CD4⁺ cells. Class I molecules are assembled by an α chain consisting of three domains and the invariant β 2-microglobulin. The α 3 domain is anchored in the cell membrane. Domains α 1 and α 2 form the peptide binding groove which is built up by α -helices. For class I, three different α chain genes exist (HLA-A, -B, and -C) (Schreuder *et al.*, 1999). Class II molecules have a similar structure as class I, but consist of two membrane anchored chains (α and β). The peptide binding groove is built up by the α 1 and β 1 domains. MHC class I molecules bind short peptides of 8-10 amino acids, whereas class II molecules can also bind longer peptides due to a more open end of the peptide binding groove. There are three pairs of MHC class II genes (HLA-DR, -DP, and -DQ).

Peptides are bound into the peptide binding groove in an extended form with the anchor residues buried in specific pockets, which are variable between different alleles. The extended binding form of the peptide makes the upward-pointing residues accessible for the interaction with the TCR. TCR bind to peptide/MHC over the center of the binding groove in a diagonal orientation relative to the peptide (Garboczi *et al.*, 1996). The V α domain is located above the N-terminal part of the peptide and V β above the C-terminal part. Contacts to the peptide are mainly mediated through the extremely variable CDR3 loops, whereas binding to MHC is made through CDR1 and CDR2 (Marrack *et al.*, 2008).

1.1.5 TCR signaling

A lot is known about the interaction of TCR α/β dimers with peptide-MHC complexes because crystal structures have been generated and analyzed. However, signal transduction from the antigen binding region of TCR α and TCR β chains to the signaling molecules (CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, $\zeta\zeta$) is still a matter of debate, which includes models proposing TCR clustering, coreceptor recruitment and/or conformational changes (Kuhns *et al.*, 2006). A recent model suggests that the movement of the T cell relative to the antigen presenting cell results in a force which acts on TCR bound to MHC/peptide complex. If antigen binding is strong enough, this force will lead to a deformation of the TCR-CD3 complex (Ma *et al.*, 2008). Changes in CD3 molecules result in an unbinding of their cytoplasmic tails from the membrane, which was shown for CD3 ϵ (Xu *et al.*, 2008). Thereby, the two key tyrosines of the ITAM are accessible to be phosphorylated by the Src-family protein kinases Fyn and Lck allowing binding of the cytosolic tyrosine kinase ZAP-70, which is then activated by Lck (Kane *et al.*, 2000). This leads to the phosphorylation of the adaptor proteins LAT and SLP-76 resulting in the activation of phospholipase C- γ . Via protein kinase C- θ , increased intracellular Ca²⁺ concentrations, and the MAP kinase cascade the transcription factors NF κ B, NFAT, and AP-1 are switched on. These transcription factors induce specific gene expression. If T cells get in addition to TCR stimulation a costimulatory

signal, e.g. via CD28, the activation results in proliferation and differentiation of T cell and the release of cytokines like interleukin (IL)-2. In cytotoxic T lymphocytes (CTL) the release of cytotoxic proteins (granzymes, perforin, and granulysin) and cytokines like interferon (IFN)- γ is triggered.

1.1.6 Trafficking of TCR

Due to their signal peptides, the components of a TCR complex are synthesized by the ribosomes directly into the endoplasmatic reticulum, where also the assembly of the complex starts. First the two heterodimers CD3 $\gamma\epsilon$ and CD $\delta\epsilon$ are formed, which then assemble with the TCR α and TCR β chains (Geisler, 2004). Finally the assembly of the TCR complex is completed in the endoplasmatic reticulum or the Golgi apparatus by adding the $\zeta\zeta$ homodimer to the hexameric complex ($\alpha\beta\gamma\epsilon\delta\epsilon$). If the $\zeta\zeta$ dimer is not correctly associated, the TCR is sorted from the Golgi apparatus to lysosomes and degraded, because of unmasked motifs of CD3 γ . TCR components, which fail to assemble in the endoplasmatic reticulum, are recognized because of their unpaired polar transmembrane residues and transported to the cytoplasm where they are degraded (Call and Wucherpfennig, 2007). Correctly assembled TCR complexes are transported to the cell surface. On resting T cells approximately 30,000 TCR are present on the cell surface (Labrecque *et al.*, 2001). TCR are not stably expressed, but circle between an inside pool located in endosomes and the cell surface. Inside the endosomes defect TCR complexes are sorted out, which leads to their degradation in lysosomes.

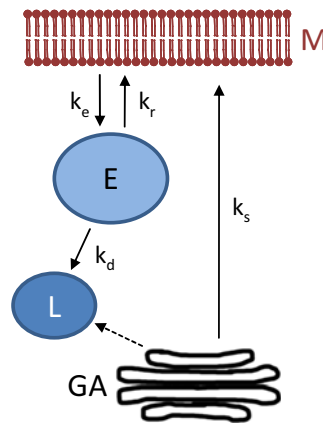


Figure 4: Model of TCR trafficking. Modified from (Geisler, 2004). Newly synthesized TCR are transported from the Golgi apparatus (GA) to the cell membrane (M). Undamaged TCR cycle between the membrane and an inside pool located in endosomes (E). Damaged or incorrectly assembled TCR are degraded in lysosomes (L). Rate constants: k_s synthesis, k_e endocytosis, k_r recycling, and k_d degradation.

Considering these points, one can design a model with following rate constants (Figure 4): new synthesis of TCR (k_s), endocytosis from the plasma membrane (k_e) recycling from the endosomes (k_r), and degradation from the endosome (k_d) (Geisler, 2004). In resting cells the number of TCR molecules on the cell surface is stable, so there is a steady state, where a number of TCR is

endocytosed and at the same time, the same number is recycled and synthesized. At steady state, 70-85% of TCR are on the cell surface and 15-30% are located inside the cell. For resting cells, k_e (endocytosis) was determined to be $\sim 0.012 \text{ min}^{-1}$ and k_r (recycling) $\sim 0.055 \text{ min}^{-1}$ (Menne *et al.*, 2002), from which a mean residence time of TCR at the cell surface of 83 min and a cycle time (endocytosis and recycling) of 100 min can be calculated. The rate constants k_s (synthesis) and k_d (degradation) are low ($\sim 0.0011 \text{ min}^{-1}$) which results in a mean life time of a TCR complex of approximately 15 h. On average, one TCR is endocytosed and recycled nine times. After stimulation of T cells (either specific by antigen recognition or unspecific by antibody binding to the TCR complex) TCR down-regulation is induced. This is mainly achieved by a three to four times increased endocytosis rate constant (k_e) (von Essen *et al.*, 2004). In addition TCR are faster degraded after phosphorylation by adding of ubiquitin, which leads to a three times shorter half-life of TCR complexes.

1.2 Adoptive T cell therapy

1.2.1 Tumors and the immune system

Influences of the immune system on tumors are a matter of debate since more than 100 years. Already at the beginning of the last century tumor transplantation experiments in animal models (mice and rats) were performed (Ehrlich, 1909) and it could be shown that some spontaneously developed tumors could be transferred to other animals. However, these early studies had the problem that no inbred animal strains could be used and therefore, MHC mismatches had a big impact on the growth of tumors in other individuals. In the 1950s and 1960s, after the generation of inbred mouse strains, it was demonstrated that mice could be immunized against tumors induced by the mutagen methylcholanthrene (Prehn and Main, 1957; Old and Boyse, 1964). For example, after resection of a transplanted tumor, some mice were protected against a second challenge with the same tumor (Foley, 1953). These results indicated the existence of “tumor specific” rejection antigens (Schreiber, 2003). Immunity after vaccination with transplantable tumors was primarily mediated by T cells (Old *et al.*, 1962). Because of the findings that tumors can be rejected by the immune system, the hypothesis of cancer immunosurveillance was developed (Burnet, 1964; Burnet, 1970), which is based on the idea that also spontaneously occurring autochthonous tumors can be recognized and destroyed by the immune system. Only (pre-)malignant cells escaping the immune system would lead to cancer. Although this idea is well accepted for virus induced tumors, the existence of immunosurveillance has to be clearly mistrusted for tumors of non-viral origin (Klein and Klein, 1977; Blankenstein, 2007). However, although there are no better data supporting this idea than 30 years ago (Qin and Blankenstein, 2004), the concept of immunosurveillance is still popular (Dunn *et al.*, 2002). Furthermore, recent data have shown that spontaneous autochthonous tumors

expressing a viral antigen as a novel cellular antigen, which can act as a rejection antigen in a transplantation system, can induce tolerance and general unresponsiveness of T cells (Willimsky and Blankenstein, 2005; Willimsky *et al.*, 2008).

Although there is no evidence that T cells are involved in the control of autochthonous non-viral tumors, there is no doubt that T cells are able to target and reject even large established tumors (Spiotto *et al.*, 2004). However, attempts to trigger the immune system of tumor patients by vaccination with peptide, proteins, DNA, or irradiated tumor cells have shown no clear success (Rosenberg *et al.*, 2004). A more promising approach is the adoptive transfer of tumor reactive T cells.

1.2.2 Adoptive T cell therapy with unmodified T cells

Transfer of donor lymphocytes to patients with recurrent chronic myelogenous leukemia (Kolb *et al.*, 1990) was a first success of adoptive T cell therapy in the late 1980s. These patients had received allogeneic hematopoietic stem cell transplantations, and were treated with donor lymphocyte transfusion after relapse. The donor T cells recognized minor histocompatibility antigens on leukemic cells and could therefore destroy them. However, this therapy is often accompanied by graft-versus-host disease, as not only the leukemic but also normal cells can be recognized. Nevertheless, donor lymphocyte transfusion was successfully applied worldwide for different forms of leukemia (Kolb *et al.*, 2004). Nowadays, the goal of the therapy is to separate the graft-versus-leukemia effect from the graft-versus-host disease.

A further successful application of T cell therapy is the transfer of Epstein-Barr virus (EBV) (Rooney *et al.*, 1995) or cytomegalovirus (CMV) (Walter *et al.*, 1995) specific T cells to prevent or to treat opportunistic virus infections in allogeneic hematopoietic stem cell transplant recipients (Berger *et al.*, 2009). Stem cell transplantation patients have dramatically reduced numbers of T cells due to the myeloablative treatment and the depletion of T cells from the donor stem cell graft. Because of the lack of T cells, normally well controlled endogenous latent or newly acquired viruses can lead to a life-threatening infection. In patients, who received an allogeneic stem cell transplant the reactivation of CMV is a big problem and is responsible for a high rate of mortality (Boeckh *et al.*, 2003). The isolation and expansion of CMV specific T cells from the stem cell donor and the subsequent transfer into the patient led to a persistent function of these cells and control of the virus with minimal toxicity (Walter *et al.*, 1995; Einsele *et al.*, 2002). For EBV, T cell deficiency can lead to uncontrolled proliferation of infected B cells. This lymphoproliferative disease could be treated by transfer of polyclonal virus specific T cells, which were isolated by *in vitro* stimulation with EBV transformed B cell lines. T cells from the stem cell donor or a third party donor were used. The transfer of EBV specific T cells could prevent lymphoproliferative diseases when used in a

prophylactic setting and in addition caused tumor regression when applied in patients with established disease (Rooney *et al.*, 1998; Burns and Crawford, 2004).

A third promising therapy with unmodified T cells was the treatment of patients with metastatic melanoma (Rosenberg and Dudley, 2009). For this therapy, tumor infiltrating lymphocytes (TIL) were isolated from the patient (Figure 5). TIL are cultured with IL-2 and several TIL lines were generated, which were tested for their function, like IFN- γ release after cocultivation with tumor cells. Functional TIL were rapidly expanded to high cell numbers ($\sim 5 \times 10^{10}$) and reinfused into the patient, who had received a lymphodepleting chemotherapy regime. Lymphodepletion before infusion of expanded cells enhanced the efficiency of the treatment (Dudley *et al.*, 2002) compared to older studies (Rosenberg *et al.*, 1988), as it enabled a better proliferation and survival of infused cells, sometimes reaching levels of 75% of all CD8⁺ cells 6-12 months after infusion. The persistence of transferred cells is of importance, as it correlates with the likelihood of tumor regression (Robbins *et al.*, 2004). Treatment of patients suffering from metastatic melanoma, which were refractory to all other treatments, resulted in an objective cancer regression in approximately 50% of patients.

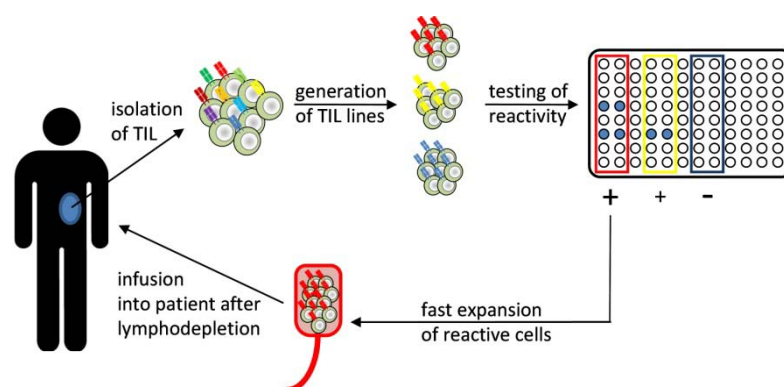


Figure 5: Adoptive cell therapy for patients with metastatic melanomas. Modified from (Dudley and Rosenberg, 2003). TIL from melanoma patients are isolated and several TIL lines are generated. Their reactivity is tested by IFN- γ release after cocultivation with tumor cells. Functional TIL lines are expanded to high numbers and reinfused into the lymphodepleted patients.

1.2.3 TCR gene therapy

Despite of good response rates when treating melanoma patients the transfer of unmodified T cells is not applicable for most other tumors, as often no TIL can be isolated. Furthermore the isolation and expansion of autologous tumor reactive T cells is time consuming and can fail also for melanomas. Therefore, the strategy of TCR gene therapy was developed, where autologous T cells are modified by transfer of TCR α and TCR β chain genes of a tumor reactive TCR. The gene transfer enables the fast generation of autologous T cells with any wanted specificity, at least if a TCR with the desired specificity exists. TCR genes can be isolated either from TIL or from T cells generated by

“reverse immunology”. For this approach, an antigen is chosen and then peripheral blood lymphocytes (PBL) from a - in most cases healthy - donor are stimulated with this antigen to induce proliferation of T cells with the desired specificity. These cells can then be enriched and cloned by limiting dilution (Wilde *et al.*, 2009). To circumvent the lack of high avidity T cells against self antigens, due to deletion in the thymus, allogeneic PBL were used (Stauss, 1999). Isolated TCR genes are cloned into an appropriate vector, most commonly based on retroviral vectors (Figure 6). In case of viral vectors, large amounts of virus supernatant can be produced and stored. Viral supernatants are used to transduce PBL from patients, which are then transferred back into the lymphodepleted patient. Figure 6 shows the ideal situation, where the transferred TCR completely replace the endogenous TCR.

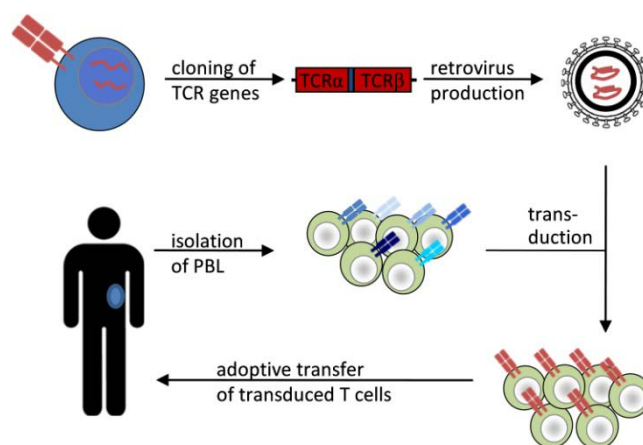


Figure 6: Principle of TCR gene therapy. TCR genes from a tumor reactive T cell are cloned into an appropriate vector and viral supernatant is produced. PBL from cancer patients are isolated and after transduction reinfused into the lymphodepleted patient.

The possibility to endow T cells with a new specificity by transferring TCR α and TCR β chain genes was first shown more than 20 years ago for a murine TCR (Dembic *et al.*, 1986). The proof that TCR redirected T cells are able to function *in vivo*, was given in a mouse model using an influenza specific TCR (Kessels *et al.*, 2001) and further mouse models were developed for preclinical studies (Bendle *et al.*, 2009). Also for the human situation, the redirection of T cells could be shown for many different antigens (Clay *et al.*, 1999; Heemskerk *et al.*, 2003; Morgan *et al.*, 2003; Engels *et al.*, 2005). In 2006, the first clinical trial for TCR gene therapy was reported (Morgan *et al.*, 2006), where two of seventeen patients responded to treatment with TCR gene modified T cells, demonstrating the clinical applicability of this approach. In this study a TCR recognizing the peptide Melan-A₂₇₋₃₅ bound to HLA-A2 was used to transduce PBL of patients with progressive metastatic melanoma. Even though transduced cells recognized their antigen *in vitro*, only in two patients a response could be observed. One reason was that gene modified T cells with Melan-A₂₇₋₃₅ specificity were rarely

detected in the patients, although reasonable transduction efficiencies were achieved. The most likely explanation for low level surface expression of the transferred TCR α/β combination on recipient T cells was the occurrence of competition with endogenous TCR and formation of mixed pairs of endogenous and exogenous TCR chains (see chapter 1.3). In a second clinical trial a TCR recognizing the same antigen (HLA-A2/ Melan-A₂₇₋₃₅) but with a higher affinity was used (Johnson *et al.*, 2009). In this study, the response rate was superior as before, with six out of twenty patients responding to the treatment. However, the treatment was accompanied with severe side effects in all responding and many non-responding patients, due to the destruction of melanocytes in skin, eye, and ear. In addition, a murine TCR directed against gp100₁₅₄₋₁₆₂ bound to HLA-A2 was used. Using this TCR, three out of sixteen patients responded to the treatment, including one complete responder. Yet, also with the gp100 specific TCR the destruction of normal melanocytes was observed.

At the moment, there is a clear discrepancy in efficiency between expanded TIL and TCR gene modified T cells. One reason could be that TIL cultures include cells with different specificities and therefore several antigens can be targeted. Among these antigens are possibly more suitable ones than Melan-A or gp100, which could also include tumor specific (see chapter 1.2.4) ones (Lennerz *et al.*, 2005). Furthermore, the presence of CD4⁺ cells could contribute to the more successful therapy with TIL (Muranski and Restifo, 2009).

A second possibility to modify T cells for an immunotherapy is the transfer of chimeric antigen receptors (CAR), which consist of the antigen binding region of an antibody and a signaling domain of TCR (Eshhar *et al.*, 1993). CAR have the advantage that they recognize their antigen in a MHC independent manner. However, a superior function of CAR modified T cells in comparison to TCR modified T cells have not been shown.

1.2.4 *Antigens for T cell therapy*

In general, one can divide possible antigens, which can be targeted by immunotherapy, into two groups, tumor specific and tumor associated antigens. Tumor specific antigens include peptides from mutated oncogenes and tumor suppressor genes and additionally viral peptides for virus derived tumors. Mutations leading to a new epitope can be point mutations resulting in an amino acid exchange (Monach *et al.*, 1995), which was shown for ras and p53 (Carbone *et al.*, 2005), frameshift mutations, which is followed by the synthesis of new polypeptides (Linnebacher *et al.*, 2001), and translocations resulting in fusion genes like BCR-ABL (Kessler *et al.*, 2006). Tumor associated antigens are non-mutated antigens overexpressed on tumor cells compared to normal cells (e.g. Her-2/neu or melanocyte differentiation antigens like Melan-A and gp100). However, targeting these antigens can cause damage of normal cells expressing the antigen. In addition humans might be tolerant against them, as they are also expressed in the thymus. A second category of tumor associated antigens are

the so called cancer/testis antigens, which are normally expressed in male germ cells (NY-ESO, MAGE (van der Bruggen *et al.*, 1991)). These cells do not express MHC molecules and can therefore not be targeted by T cells. However, since cancer/testis antigens are expressed in the thymus (Gotter *et al.*, 2004), they also might induce tolerance. Therefore, they should be better called cancer/testis/thymus antigens (Preiss *et al.*, 2005).

Independent of the choice for a tumor associated or tumor specific antigen, it seems to be important for efficient tumor elimination that the antigen is cross-presented on cells of the tumor stroma (Spiotto *et al.*, 2004; Yu *et al.*, 2006).

The selection of an antigen targeted by adoptive T cell therapy has to be well reflected (Offringa, 2009), as the choice of a wrong (tumor associated) antigen can lead to severe side effects in patients (Johnson *et al.*, 2009) and a failure of the therapy.

1.3 Dual TCR T cells

When TCR genes are transferred into T cells (as described in chapter 1.2.3), T cells evolve that can theoretically express four different TCR α / β combinations on their surface: the endogenous, the exogenous, and two mixed TCR α / β combinations consisting of endogenous and exogenous TCR chains (Figure 7).

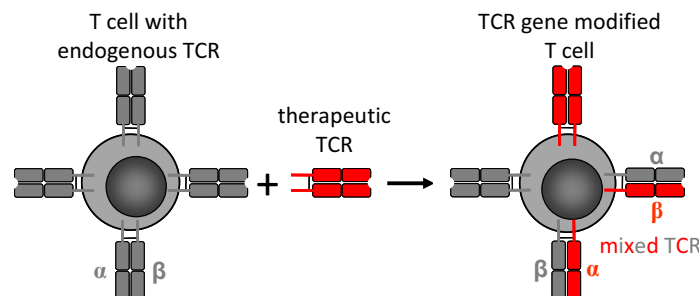


Figure 7: After transduction of T cells four different TCR α / β combinations can be expressed. The generation of tumor reactive T cells for adoptive therapy involves the transfer of one TCR α and one TCR β chain gene into T cells, which already express an endogenous TCR. By random combination of the TCR chains, four different TCR α / β combinations are possible.

The clonal selection theory in the 1950s postulated that one lymphocyte has one specific antigen receptor (Talmage, 1957; Burnet, 1959). This dogma was first verified for B cells (Nossal and Lederberg, 1958) and later also accepted for T cells. However, in the 1990s, T cells with either two TCR α (Padovan *et al.*, 1993; Heath *et al.*, 1995) or two TCR β (Davodeau *et al.*, 1995; Padovan *et al.*, 1995) chains were shown to exist in humans as well as in mice, indicating that some T cells express more than one TCR. The expression of only one specific TCR per T cell is normally guaranteed by a defined order of TCR α and TCR β chain locus rearrangements during T cell development in the

thymus. First, one TCR β chain locus is rearranged followed by expression of the TCR β chain together with the constant pre-TCR α chain on the cell surface (Saint-Ruf *et al.*, 1994). This triggers cell proliferation and stops rearrangement of other TCR β chain genes simultaneously. Next, the TCR α chain locus is rearranged. However, due to incomplete allelic exclusion, T cells exist, which can express more than one TCR. As this happens relatively often for TCR α , it was proposed that up to 30% of human T cells have two rearranged TCR α gene loci and could express two different TCR on the surface (Padovan *et al.*, 1993). These naturally occurring dual TCR T cells might contribute to the high proportion of alloreactive T cells (Morris and Allen, 2009).

It is still not clear if clonality of T cells is regulated only at the genetic level by allelic exclusion, or if also other mechanisms play a role. Posttranslational mechanisms were suggested as on the surface of T cells, which were transgenic for two TCR, either one or the other TCR was found, although both TCR α and TCR β chains were expressed inside the cells (Sant'Angelo *et al.*, 2001). In other models using T cells with two transgenic TCR only function via one TCR was detected, due to the lack of one TCR α chain on the cell surface. However, on these cells, both TCR β chains were expressed (Lacorazza and Nikolich-Zugich, 2004). In this and a further model, a posttranslational mechanism was suggested, which accomplishes clonality in cells expressing two TCR α chains by suppressing one of them. It was shown that protein tyrosine kinase Lck and TCR activation were involved in this mechanism (Niederberger *et al.*, 2003). In contrast, in other models double transgenic T cells expressing two functional TCR occurred (Zal *et al.*, 1996; Gladow *et al.*, 2004; Weinhold *et al.*, 2007). The pairing of endogenous and exogenous chains bears the risk that the arising new specificities may be directed against self-antigens and lead to autoimmunity, which was already observed in a mouse model (Bendle *et al.*, 2009). This becomes more likely, when T cells expressing many different endogenous TCR are transduced. Moreover, such TCR are not controlled by selection mechanisms in the thymus. A second possible problem of dual TCR T cells is the activation of tolerant cells by stimulation via the introduced TCR. Both problems may be limited by using virus specific T cells for transduction (Heemskerk *et al.*, 2004). However, for this approach an extensive *ex vivo* cultivation of cells would be needed. To eliminate transduced cells, which are autoreactive and cause graft-versus-host disease, safety mechanisms were developed. For example a small tag, was introduced into the TCR. Cells could be destroyed by adding depleting antibodies (Ab) recognizing the tag (Kieback *et al.*, 2008). A second strategy to eliminate cells was the transfer of suicide genes (Vassaux and Martin-Duque, 2004). However, using these strategies results in a depletion of all transferred cells and therefore terminates the therapy.

Beside the risk of autoimmunity, the expression of TCR molecules with different specificities on one cell reduces the expression level of the desired TCR and therefore may affect function of T cells.

As there is no consistent picture about TCR expression on dual TCR T cells and in addition most experiments had been made with transgenic animals and not using *in vitro* TCR gene transfer, it is important to examine the mutual influence of two TCR in well controlled models.

1.4 Optimizing TCR for TCR gene therapy

1.4.1 Optimizing the affinity

The term affinity will be used, when the binding strength of one TCR to one MHC/peptide complex is meant. To increase the affinity of TCR, yeast (Holler *et al.*, 2000) or phage (Li *et al.*, 2005) displays were successfully used for screening of random mutations. Furthermore, TCR with higher affinities could be identified after directed mutagenesis of CDR3 regions and screening of the variants after transfection into T cells (Robbins *et al.*, 2008). However, modifications of CDR3 regions always include the risk of losing specificity (Zhao *et al.*, 2007). In addition, the optimal affinity of a TCR used for therapy is still a matter of debate, and the need for TCR with affinities higher than naturally appearing ones has not been shown until now.

1.4.2 Optimizing the avidity

The term avidity will be used, when the sum of all bindings between a T cell and an antigen presenting cell is meant. During the last years, several strategies were developed to increase the avidity of transduced T cells by increasing the TCR level on the cell surface (Uckert and Schumacher, 2009). First, this was achieved utilizing methods to attain a high level of transgene expression by improving gene transfer systems, most commonly based on retroviral vectors. This included the development of retroviral envelope proteins (Miller and Rosman, 1989; Bunnell *et al.*, 1995; Uckert *et al.*, 2000), modifications of regulatory elements (Engels *et al.*, 2003), and arrangement of the TCR gene cassette (Leisegang *et al.*, 2008). Further improvement included optimization of TCR encoding nucleotide sequences (codon-optimization), which involved the replacement of infrequently used codons and the deletion of (cryptic) splice sites and RNA instability motifs. Codon-optimization resulted in enhanced translation of the transgenes (Scholten *et al.*, 2006). However, these approaches only led to higher TCR protein levels but did not impact on preferential pairing of transferred TCR chains. Therefore, strategies were also investigated to obtain improved pairing. First, molecular design of the C-region TCR α/β interface was tried by exchanging small and large amino acids between the two chains (Voss *et al.*, 2008). This resulted in reduced expression of mixed TCR, but did not yield T cells with higher functional avidity as compared to cells transduced with unmodified TCR. Second, the exchange of the original C-domains downstream of the interchain disulfide bond by the complete human TCR ζ chain was employed (Sebestyen *et al.*, 2008). This

modification completely eliminated the formation of mixed TCR, as pairing between modified and wild type TCR chains was no longer possible, but a functional advantage for the modified cells still needs to be shown. Third, mutations of single amino acids to cysteines in the immunoglobulin like domains of each C-region led to formation of an additional disulfide bond connecting the C-regions of the TCR α and TCR β chains (Cohen *et al.*, 2007; Kuball *et al.*, 2007), which most probably stabilized the TCR and impeded the binding to wild type TCR chains. This led to a reduced amount of mixed TCR and to an improvement of the functional avidity of TCR gene modified cells. Fourth, independent of these modifications to improve the surface expression of transferred TCR, it was shown that substitution of human TCR C-regions by corresponding murine counterparts increased the cell surface expression of these hybrid TCR compared to wild type human TCR. Furthermore, when murine TCR C-regions were replaced by their human counterparts, a decreased expression of the hybrid TCR was noted in comparison to wild type mouse TCR (Cohen *et al.*, 2006; Voss *et al.*, 2006). This rather unexpected observation subsequently led to the concept of “murinization”, in which both C-regions of human TCR are exchanged by murine C-regions to achieve a higher functional avidity of TCR gene modified human T cells (Cohen *et al.*, 2006). Furthermore, the occurrence of mixed TCR was reduced, as human and murine C-regions bound less efficiently to each other. The reason, why murine TCR were better expressed was not known and in addition it was not clarified if the complete murine C-regions or only parts are needed.

The different optimization strategies had been demonstrated only by few examples and in addition were not compared to other strategies. Therefore, an evaluation of different options in a direct comparison with more TCR is needed.

Table 1: Summary of different TCR optimization strategies.

Optimization strategy	Abbr.	Pro	Contra	Ref.
<i>in vitro</i> evolution		increased affinity	risk of loss of specificity	(Holler <i>et al.</i> , 2000)
codon-optimization	co	higher protein levels, no changes of amino acid sequence	no preferential pairing, completely new nucleotide sequence	(Scholten <i>et al.</i> , 2006)
murinization	mu	preferential pairing, improved functional avidity	immunogenicity is likely due to complete mouse gene segments	(Cohen <i>et al.</i> , 2006)
additional disulfide bond	cys	preferential pairing, only small changes of amino acid sequence, improved functional avidity	even one amino acid exchange can be immunogenic	(Cohen <i>et al.</i> , 2007; Kuball <i>et al.</i> , 2007)
amino acid exchanges between TCR α and TCR β		slightly reduced mispairing	no functional improvement	(Voss <i>et al.</i> , 2008)
TCR ζ hybrid		no mispairing	no functional improvement, altered TCR function cannot be excluded	(Sebestyen <i>et al.</i> , 2008)

2 Tasks of this thesis

- ➔ TCR gene transfer is an established method to create T cells with desired antigen specificities. After gene transfer, the transferred TCR has to compete with the endogenous TCR. As it was not known, how the two TCR influence each other, the first task of this thesis was to establish human and murine models to examine the mutual influence of the two TCR. The models should have the possibilities to characterize both TCR by Ab and multimer staining and to perform functional assays.
- ➔ While working on the first task, we found that some (weak) TCR are hardly expressed on the cell surface. To enable a sufficient expression level of these TCR, the second task was to compare different published TCR optimization strategies and to get an insight which modifications are needed to gain functional T cells after TCR gene transfer. Several TCR should be cloned in different versions and transduced into primary human cells. Expression levels of the different TCR and function of transduced T cells should be analyzed.
- ➔ A promising optimization strategy for the improved functional expression of human TCR is the so called murinization, where the human C-regions are exchanged by their murine counterparts. However, it is likely that the use of complete murine gene segments leads to immunogenicity of these hybrid TCR. Therefore, the third task was to identify those amino acids within the murine C-regions, which are responsible for the improved functional expression of murinized TCR. To achieve this, a model cell line had to be established, with which different human/murine hybrid TCR could be tested. Afterwards, the TCR constructs with the identified murine amino acids, which are responsible for the improved expression, had to be tested with different TCR on primary human cells. The expression level should be analyzed by multimer staining. In addition function of transduced cells had to be examined in comparison to cells transduced with wild type or completely murinized TCR.

3 Materials and Methods

3.1 Materials

3.1.1 Cells

293T (ATCC: CRL-11268, American Type Culture Collection, Manassas, USA), 293(HLA-A2⁺) cells and packaging cell lines 293-10A1 (Farson *et al.*, 1999), Plat-E (Morita *et al.*, 2000), and GP+E86 (Markowitz *et al.*, 1988) were cultured in Dulbecco's modified Eagle's medium (D-MEM, GIBCO, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany). The renal cell carcinoma (RCC) cell line RCC-26 (Schendel *et al.*, 1993) was cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 1 mM sodium pyruvate (GIBCO), and 100 U/ml penicillin/streptomycin. Melanoma lines, Mel-A375 (HLA-A2⁺, tyrosinase⁻) and Mel-624.38 (HLA-A2⁺, tyrosinase⁺) (Rivoltini *et al.*, 1995) were cultured in RPMI 1640 medium supplemented with 12% FCS, 1 mM sodium pyruvate, and 1% amino acids solution (MEM, GIBCO). Human PBL, human T cell lines Jurkat76 (J76) (Heemskerk *et al.*, 2003), J76/TCR26, J76/TCR53, HUT78 (ATCC; TIP-161), the murine T cell line 58 $\alpha\beta$ ⁻ (Letourneur and Malissen, 1989), the chicken ovalbumin (OVA) specific T cell hybridoma B3Z (Karttunen *et al.*, 1992), TAP deficient HLA-A2⁺ T2 cells (ATCC: CRL-1992), and human B lymphoblastoid cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS (PAN Biotech, Aidenbach, Germany), 1 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 100 U/ml penicillin/streptomycin. Human CMV specific CTL line T21 (V β 20) and the Melan-A specific CTL clone InRi1 (V α 2, V β 14) (Fleischer *et al.*, 2004) were cultured with RPMI 1640 medium supplemented with 10% human serum (Valley Biomedical, Winchester, USA), 100 U/ml penicillin/streptomycin, 50 U/ml IL-2 (Chiron, Marburg, Germany), 2 ng/ml IL-15 (Sigma-Aldrich, Munich, Germany), and 10 ng/ml anti-CD3 monoclonal antibodies (mAb) (Janssen-CILAG, Neuss, Germany) for stimulation. As feeder cells, 2.5x10⁷ allogeneic peripheral blood mononuclear cells (PBMC) (irradiated with 30 Gy) and 5x10⁶ B lymphoblastoid cell lines (irradiated with 100 Gy) from healthy donors were added per T30 tissue culture flask (~10 ml). All cell culture flasks and plates were purchased from BD Falcon (Franklin Lakes, USA).

PBMC were isolated from blood of healthy donors with donors' informed consent by ficoll gradient centrifugation and stimulated in non-tissue culture 24-well plates, precoated with anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml) mAb (BD Pharmingen, Heidelberg, Germany). Cells were seeded in a concentration of 1x10⁶ per well and ml and 100 U/ml IL-2 were added. PBL were cultured with 100 U/ml IL-2 until day thirteen after isolation and then rested for two days by reducing the IL-2 concentration to 10 U/ml.

J76 and 58 $\alpha\beta^-$ cells are TCR α and TCR β deficient but express all other TCR components. Cell lines J76/TCR26 and J76/TCR53 were generated by transduction of J76 cells with TCR26 (Engels *et al.*, 2005) and TCR53, respectively, encoding vectors (MP71-TCR26- β -IRES- α , MP71-TCR53- β -IRES- α) and enriched for TCR expressing cells by MACS (cells were provided by M. Leisegang). B3Z cells are transfected to express β -galactosidase under the minimal human IL-2 promoter; β -galactosidase expression is induced through binding of the OVA specific TCR (V α 13, V β 5) to MHC(H2-K^b)-ova₂₅₇.

3.1.2 Peptides

Peptides SIINFEKL (ova₂₅₇), KAVYNFATM (gp₃₃), SIYRYYGL (SIY), IMDQVPFSV (gp100), NLVPMVATV (CMV), SLLMWITQV (NY-ESO-1), YMDGTSMSQV (tyrosinase), and AAGIGILTV (Melan-A) were purchased as HPLC purified products (Biosyntan, Berlin, Germany). Amino acids in bold were modified compared to wild type peptides to prevent dimerisation of peptides or to enhance binding to MHC molecules.

3.1.3 Antibodies for staining

Table 2: List of mAb directed against murine (m) and human molecules.

mAb	clone	conjugated to	isotype
mTCRV α 2	B20.1	APC	rat IgG _{2a} , λ
mTCRV β 5.1/5.2	MR9-4	FITC	mouse IgG ₁ , κ
mTCRV β 8.1/8.2	MR5-2	PE	mouse IgG _{2a} , κ
mTCRC β	H57-597	APC	hamster IgG ₂ , λ 1
CD3	UCHT1	PE	mouse IgG ₁ , κ
CD8	HIT8a	FITC	mouse IgG ₁ , κ
TCRV β 4	WJF24	PE	rat IgM
TCRV β 8	56C5	PE	mouse IgG _{2a}
TCRV β 20	ELL1.4	PE	mouse IgG _{2a}
TCRV β 22	IMMU 546	FITC, PE	mouse IgG ₁

Ab had been conjugated to allophycocyanin (APC), phycoerythrin (PE) or fluoresceinisothiocyanat (FITC). Antibodies directed against human TCRV β -regions were bought from Immunotech (Marseille, France), all other antibodies were purchased from BD Pharmingen.

3.1.4 MHC-multimers

MHC multimers are based on recombinant MHC molecules. These molecules are folded with the peptide of interest, multimerized and fluorescently labeled. Multimers specifically label T cells that express TCR specific for the used peptide-MHC complex. APC-labeled MHC-tetramers loaded with gp100 and tyrosinase peptide, respectively, were purchased from BeckmanCoulter (Krefeld, Germany) and APC-labeled MHC-pentamers loaded with NY-ESO peptide were bought from Proimmune (Oxford, UK). PE-labeled MHC-tetramers loaded with CMV and Melan-A peptide,

respectively, and reversible gp100-multimers (Knabel et al., 2002) were provided by D. Busch (Technical University Munich, Germany).

3.1.5 Primers

Table 3: Primers used for cloning.

Name	Sequence	Used for
fwd	CCCTCTCTCCAAGCTCACTT	cloning from pMP71
rev	CAATATGGAATAAATGGCGGTAAGATGC	
r_C α _EcoRI	TGGAATTCTCAGCTGGACCACAGCCGACGC	cloning of TCR α chains
r_C β 1_EcoRI	TGGAATTCTCAGAAATCCTTCTCTTGACC	cloning of TCR β (C β 1) chains
r_C β 2_EcoRI	TGGAATTCCTAGCCTCTGGAATCCTTCTC	cloning of TCR β (C β 2) chains
f_V α 3_NotI	AGGCGGCCGCCACCATGGAACTCTCCTGGGA	cloning of TCR α (V α 3) chains
f_V α 7_NotI	AGGCGGCCGCCACCATGTGGGGAGTTTCTCTT	cloning of TCR α (V α 7) chains
f_V α 16_NotI	CTGCGGCCGCCATGGCCTCTGCACCCATCT	cloning of TCR α (V α 16) chains
f_V α 19_NotI	CTGCGGCCGCCATGGTGAAGATCCGG	cloning of TCR α (V α 19) chains
f_V α 22_NotI	CTGCGGCCGCCATGAACTATTCTCCAGGCT	cloning of TCR α (V α 22) chains
f_V β 4_NotI	CTGCGGCCGCCATGCTGAGTCTTCTGCTCC	cloning of TCR β (V β 4) chains
f_V β 8_NotI	CTGCGGCCGCCATGGACTCCTGGACCT	cloning of TCR β (V β 8) chains
f_V β 14_NotI	CTGCGGCCGCCATGGGCCCCAGCTCCTTG	cloning of TCR β (V β 14) chains
f_V β 20_NotI	GTGCGGCCGCCACCATGCTCTGCTCTCTCC	cloning of TCR β (V β 20) chains
f_V β 23_NotI	AGGCGGCCGCCACCATGCTTAGTCTGACCTG	cloning of TCR β (V β 23) chains
f_C α _cys	ATATCACAGACAAATGTGTGCTAGACATGA	mutation from T to C at position 47 of C α
r_C α _cys	TCATGTCTAGCACACATTTGTCTGTGATAT	
f_C β _cys	TGCACAGTGGGGTCTGCACAGACCCGACGC	mutation from S to C at position 57 of C β
r_C β _cys	GCTGCGGGTCTGTGCAGACCCCACTGTGCA	
f_C α co_cys	ACATCACCGACAAGTGCCTGTCTGGACATGC	mutation from T to C at position 47 of C α co
r_C α co_cys	GCATGTCCAGCACGCACTTGTCTGGTGTATGT	
f_C β co_cys	TGCACAGCGGCGTCTGCACCGACCCCAAGC	mutation from S to C at position 57 of C β co
r_C β co_cys	GCTGGGGGTCTGGTGCAGACGCCGCTGTGCA	
f_C α mu_cys	TCATCACTGACAAATGTGTGCTGGACATGA	mutation from T to C at position 47 of C α mu
r_C α mu_cys	TCATGTCCAGCACACATTTGTCTAGTGATGA	
f_C β mu_cys	TCCACAGTGGGGTCTGCACGGACCCCTCAGG	mutation from S to C at position 57 of C β mu
r_C β mu_cys	CCTGAGGGTCTGGTGCAGACCCCACTGTGGA	
f_C α _mu	ATCCAGAACCCTGAACCTGCCGTGTACCAG	murinization of C α
r_C α _mu	CTGGTACACGGCAGGTTTCAAGGTTCTGGAT	
f_C β _mu	GAGGACCTGAGAAACGTGACTCCACCCAAG	murinization of C β
r_C β _mu	CTTGGGTGGAGTCACGTTTCTCAGGTCCTC	
f_C α co_mu	CCAGAACCCCGAGCCCGCCGTGTACCA	murinization of C α co
r_C α co_mu	TGGTACACGGCGGGCTCGGGGTTCTGG	
f_C β co_mu	CTGAGGAACGTGACCCCCCAAGGTG	murinization of C β co
r_C β co_mu	CACCTTGGGGGGGGTACGTTCTCTCAG	
f_P2A	AGGAAAACCCTGGGCCCCATG	cloning from P2A constructs
r_P2A	GCCAGGGTTTTCTCCA	
r_P2A_C β 1	TTCCACGTCTCTGCTTCTTAACAGAGAGAAGTTCGT	cloning of TCR β (C β 1) chains as

	GGCGCCGCTTCCGAAATCCTTTCTCTTGACCATGGCCATCAA	P2A constructs
r_P2A_C β 2	TTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCCGCTTCCGCTCTGGAATCCTTTCTCTTGACC	cloning of TCR β (C β 2) chains as P2A constructs
r_P2A_C β 2_co	CTCCACGTGCGCCGCTGCTTCAGCAGGCTGAAGTTGGTGGCGCCGCTGCCGCCCCGCTGTCTTCCGCTTCACC	cloning of TCR β co (C β 2) chains as P2A constructs
r_P2A_C β 2_mu	GGGCCCAGGGTTTTCTCCACGTGCGCCGCTGCTTCA GCAGGCTGAAGTTGGTGGCGCCGCTGCCGCTGTTCTTCTTTCACCATGGCCA	cloning of TCR β mu (C β 2) chains as P2A constructs
r_P2A_C β 2_muco	GGGCCCAGGGTTTTCTCCACGTGCGCCGCTGCTTCA GCAGGCTGAAGTTGGTGGCGCCGCTGCCGCTGTTCTTCTTTCACCATGGCCA	cloning of TCR β muco (C β 2) chains as P2A constructs
f_P2A_V α 3	TGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGGAACTCTCCTGGGAGTGTCTTTGGTG	cloning of TCR α (V α 3) chains as P2A constructs
f_P2A_V α 7	TGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGTGGGGAGTTTTCTTCTTTATGTTTCC	cloning of TCR α (V α 7) chains as P2A constructs
f_P2A_V α 16	AATTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGA AAACCCCGGTCCCATGGCCTCTGCACCCATCTCGATG	cloning of TCR α (V α 16) chains as P2A constructs
f_P2A_V α 16_co	AATTCTCAGCTGCTGAAGCAGGCCGCGCAGCTGGAGG AAAACCCCTGGCCCA7GGCCAGCGCCCCATCAGCATG	cloning of TCR α co (V α 16) chains as P2A constructs
f_P2A_V α 19	AATTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGA AAACCCCGGTCCCATGGTGAAGATCCGGCAATTTTGT	cloning of TCR α (V α 19) chains as P2A constructs
f_P2A_V α 19_co	GACGTGGAGGAAAACCCCTGGCCCA7GGTGAAGATCCGGCAGTT	cloning of TCR α co (V α 19) chains as P2A constructs
f_P2A_V α 22	TGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGAACTATTCTCCAGGCTTAGTATCTCTG	cloning of TCR α (V α 22) chains as P2A constructs
f_C β _2	TACTGCCTGAGCAGCCGCTGAGGGTCTC	exchange between C β domains 2 and 3
r_C β _2	GAGACCCTCAGGCGGCTGCTCAGGCAGTA	
f_C β _3	GCCGAGGCTGGGGCAGAGCAGACTGTGG	exchange between C β domains 3 and 4
r_C β _3	CCACAGTCTGCTCTGCCCCAGGCCTCGGC	
f_C β _1.1	TGTTTGAGCCATCAAAAGCAGAGAT	exchange between C β domains 1.1 and 1.2
r_C β _1.1	ATCTCTGCTTTTGATGGCTCAAACA	
f_C β _1.2	CAAAAGGCTACACTGGTGTGCCTGGCCA	exchange between C β domains 1.2 and 1.3
r_C β _1.2	TGGCCAGGCACACGAGTGTAGCCTTTTG	
f_C β _4.1	GGGTTCTGTCTGCCACCATCCTCTATGAG	exchange between C β domains 4.1 and 4.2
r_C β _4.1	CTCATAGAGGATGGTGGCAGACAGAACCCC	
f_C β _KE	TTTGAGCCATCAGAAAGCAGAGAT	mutation from K to E at position 18 of C β
r_C β _KE	ATCTCTGCTTCTGATGGCTCAAA	
f_C β _AS	TTTGAGCCATCAAAAGCAGAGATTCCAACAAA	mutation from A to S at position 22 of C β
f_C β _NH	TTTGAGCCATCAAAAGCAGAGATTGCACACAAACA	mutation from N to H at position 23 of C β
f_C β _KT	TTTGAGCCATCAAAAGCAGAGATTGCAAACACCCAAAA GG	mutation from K to T at position 24 of C β
f_C β _EK_SA	CCATCAAAAGCAGAGATCGCACACACC	mutation from E to K and S to A at position 18 and 22 of C β
r_C β _EK_SA	GGTGTGTGCGATCTCTGCTTTTGATGG	
f_C β _ER	TGTTTGAGCCATCAAGAGCAGAGATT	mutation from E to R at position 18 of C β
r_C β _ER	AATCTCTGCTTCTGATGGCTCAAACA	

f_α ₁	ATCACTGACAAA ACT GTGCTAGACATGA	exchange between α domains 1 and 2
r_α ₁	TCATGTCTAGCACAGTTT GT CAGTGAT	exchange between α domains 1 and 2
f_α _{1a}	CCAGAAAGTTCCTGTGATGCCACGTTGACT	exchange between α domains 2 and 3
r_α _{1a}	AGTCAACGTGGCATCACAGGA ACTTT CTGG	exchange between α domains 2 and 3
f_α _{1b}	TCAGACGTTCCCTGTGATGTCAAGCTGGTC	exchange between α domains 2 and 3
r_α _{1b}	GACCAGCTTGACATCACAGGGAACGTCTGA	exchange between α domains 2 and 3
f_α _{2.2.3}	GAAGACACCTTCTTCC CC CAGC	exchange between α domains 2.2.2 and 2.2.3
r_α _{2.2.3}	GCTGGGGAAGAAGGTGTCTTC	

Characters in bold represent mutations, start codons are in italic, and restriction sites are underlined. First characters of primer names describe the direction of primers, f: forward, r: reverse. Primers were synthesized by MWG (Martinsried, Germany).

3.2 Methods

3.2.1 Isolation of TCR genes

To identify V-region usage of different TCR chains, parts of TCRα and TCRβ chains, encoding CDR3 and the 3'-end of the V-region, were amplified by PCR using a panel of TCRVα and TCRVβ primers in combination with respective C-region primers (Steinle *et al.*, 1995) and sequenced (V-region identification was done in cooperation with H. Pohla, Munich).

For molecular cloning, standard protocols were used (Sambrook and Russell, 2000; Mülhardt, 2008). RNA of T cell clones was isolated (RNeasy MiniKit, Qiagen, Hilden, Germany) and transcribed to cDNA using random primers (Promega, Mannheim, Germany) and superscript-revertase (Invitrogen, Karlsruhe, Germany). Forward primers specific for the 5'-end of the V-region with an overhang encoding a *NotI* restriction site and reverse primers specific for the 3'-end of the C-region with an overhang encoding an *EcoRI* restriction site were used for a RT-PCR. PCR products and the retroviral vector plasmid pMP71 (Figure 8) (Engels *et al.*, 2003) were digested with *NotI* and *EcoRI* restriction enzymes (Fermentas, St. Leon-Rot, Germany). Subsequently, vector plasmids were dephosphorylated with alkaline phosphatase (Roche, Mannheim, Germany). Then, DNA fragments were separated by agarose-gel-electrophoresis and isolated from gel pieces with DNA Purification Kit (Biozym, Oldendorf, Germany). DNA fragments were ligated with rapid DNA ligation kit (Roche) and competent Mach1™ *E.coli* (Invitrogen) were transformed with resulting plasmids. Plasmid DNA was isolated (Invisorb Spin Plasmid Mini Two, Invitek, Berlin, Germany) and correctness of plasmids was confirmed by restriction analysis (Fermentas) and sequencing (MWG). Then larger amounts of plasmid DNA were isolated (Plasmid Midi Kit or Plasmid Maxi Kit, Qiagen).

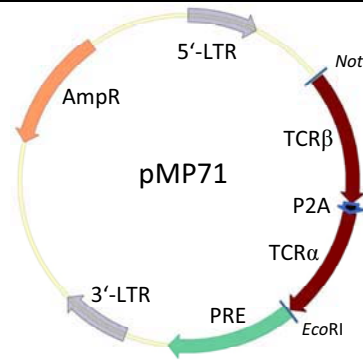


Figure 8: The retroviral vector plasmid pMP71. Genes can be cloned into the retroviral vector plasmid pMP71 via *NotI* and *EcoRI* restriction sites. Genes are followed by the posttranscriptional regulatory element (PRE) of the woodchuck hepatitis virus and flanked by the long terminal repeats (LTR) of the myeloproliferative sarcoma virus. In addition, the plasmid contains an ampicillin resistance gene (AmpR). In this example, genes (TCR β and TCR α) are linked via a peptide linker (P2A).

To ensure simultaneous expression of both TCR chains, genes were fused via a 2A peptide (P2A) (Szymczak *et al.*, 2004). The 5' gene was amplified using a reverse primer and the 3' gene using a forward primer including overlapping parts of the P2A sequence. The 5' genes were amplified excluding the stop codon. Fragments were annealed and cloned as described in chapter 3.2.2.

Table 4: TCR used for transduction experiments.

TCR	recognized peptide	TRAV	TRAJ	V α (Arden)	TRBV	TRBJ	TRBC	V β (Arden)
P14	LCMV-gp33	14D-1*01	48*01	2.4	13-3*01	2-4*01	2	8.1
gp100-TCR	gp100 ₂₀₉₋₂₁₇	41*01	54*01	19.1	12-3*01	2-1*01	2	8.1
NY-ESO-1-TCR	NY-ESO ₁₅₇₋₁₆₅	3*01	28*01	16.1	29-1*01	2-7*01	2	4.1
TCR53	?*	41*01	13*02	19.1	30*01	2-5*01	2	20.1
T58	tyrosinase ₃₆₉₋₃₇₇	1-2*01	28*01	7.2	13*01	1-4*01	1	23.1
D115	tyrosinase ₃₆₉₋₃₇₇	9-2*03	28*01	22.1	12-4*01	2-5*01	2	8.2
IVS-B	tyrosinase ₃₆₉₋₃₇₇	17*01	47*01	3.1	27*01	2-1*01	2	14.1

All TCR recognize peptides bound to HLA-A2. In the following, nomenclature for TCR V segments of Arden will be used (Arden *et al.*, 1995). *TCR53 recognizes a shared tumor associated, yet unidentified, antigen presented on a majority of HLA-A2⁺ RCC and some other tumor cells.

TCR, which were cloned into pMP71 are summarized in Table 4. The murine P14-TCR (Pircher *et al.*, 1987) had been constructed as single TCR gene vectors (Sommermeyer, 2004). The human gp100-TCR ((Morgan *et al.*, 2003) clone R6C12) was cloned as single TCR gene vectors (TCR genes were provided by R. Morgan, National Institutes of Health, Bethesda, USA). After preliminary testing with

single TCR gene vectors, TCR D115 (Wilde *et al.*, 2009), T58 (Wilde *et al.*, 2009), and IVS-B (Wolfel *et al.*, 1993) were cloned as P2A vectors (Figure 8) either unmodified (wt, wild type) or murinized and codon-optimized (muco). In addition, TCR T58 was cloned in the versions murinized (mu) and minimally murinized (mm) as P2A vectors. For NY-ESO-1-TCR (NY-TCR, (Kronig *et al.*, 2009) clone ThP2) constructs α wt, β wt, α mu, β mu, β wt-P2A- α wt, β mu-P2A- α mu, β co-P2Aco- α co, β cys-P2A- α cys, β mucys-P2A- α mucys, β cysco-P2Aco- α cysco, β muco-P2Aco- α muco, β h1, β h2, β h3, β h4, β m1/4, β m1.2/4.1, β m1.2KE/4.1, β m1.2AS/4.1, β m1.2NH/4.1, β m1.2KT/4.1, β mm, β m1.K/4.1, β m1.KA, β m1.K, β m1.RA/4.1, α m2, α mm, β mm-P2A- α mm and for TCR53 constructs α wt, β wt, β wt-P2A- α wt, β mu-P2A- α mu, β co-P2Aco- α co, β cys-P2A- α cys, β muco-P2Aco- α muco, β mm-2A- α mm, β wt-P2A- α mu, β mu-P2A- α wt, β mm-P2A- α wt, β wt-P2A- α mm were cloned.

3.2.2 Construction of TCR gene variants

Introduction of a mutation

To introduce a mutation into a gene, two successive PCR steps were performed. First, two parts of the gene were amplified; the first part was amplified using a forward primer binding to the plasmid 5' of the gene (fwd) and a reverse primer of approximately 20 nucleotide length including the mutation (e.g. r_C α _cys). The second part was amplified using a forward primer including the mutation (e.g. f_C α _cys) and a reverse primer binding to the plasmid 3' of the gene (rev) (Figure 9a).

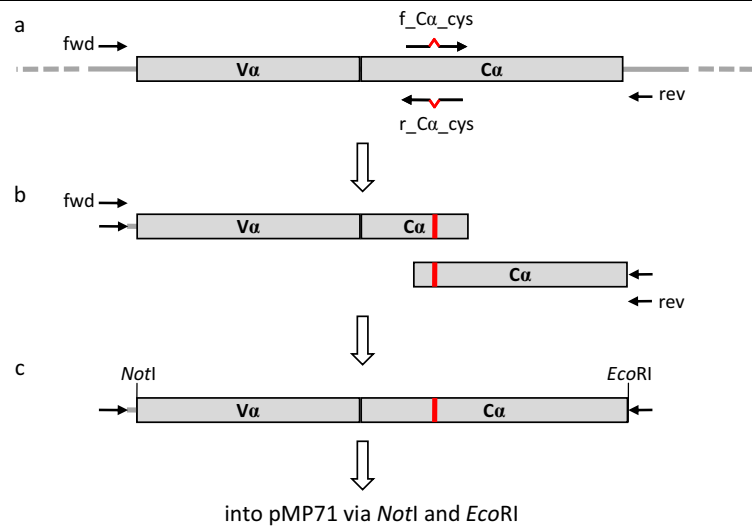


Figure 9: Introduction of a cysteine mutation into a TCR α gene. The construction of the TCR α variant with an additional cysteine is shown as an example for the introduction of a mutation. (a) Two fragments of the TCR α gene were amplified via PCR using complementary primers, which contained the mutation (f_C α _cys and r_C α _cys). (b) Complementary sequences were annealed and the complete mutated TCR α gene was amplified. (c) Finally the gene was cloned into the plasmid pMP71 via NotI and EcoRI restriction sites.

Second, the two parts of the mutated gene were combined by annealing of the 20 nucleotide long complementary sequences and subsequently amplified using the forward primer of the first PCR

(fwd) and the reverse primer of the second PCR (rev) (Figure 9b). Finally, the generated gene was integrated into the retroviral vector plasmid pMP71 via restriction sites *NotI* and *EcoRI* (Figure 9c).

Exchange of domains

If possible domains of two similar genes were exchanged using a restriction site located between the two domains. For most constructs this approach was not possible, therefore, a cloning strategy similar to the one described for mutations was used. Desired fragments of the two genes were amplified via PCR using complementary primers (e.g. f_C β _3 and r_C β _3) binding to the exchange region in combination with primers rev and fwd, respectively (Figure 10a). Subsequently the two PCR products were combined by annealing of the complementary sequences and then the complete gene was amplified via PCR with the two primers fwd and rev (Figure 10b). The gene was finally cloned into the retroviral vector plasmid pMP71 using *NotI* and *EcoRI* restriction sites (Figure 10c).

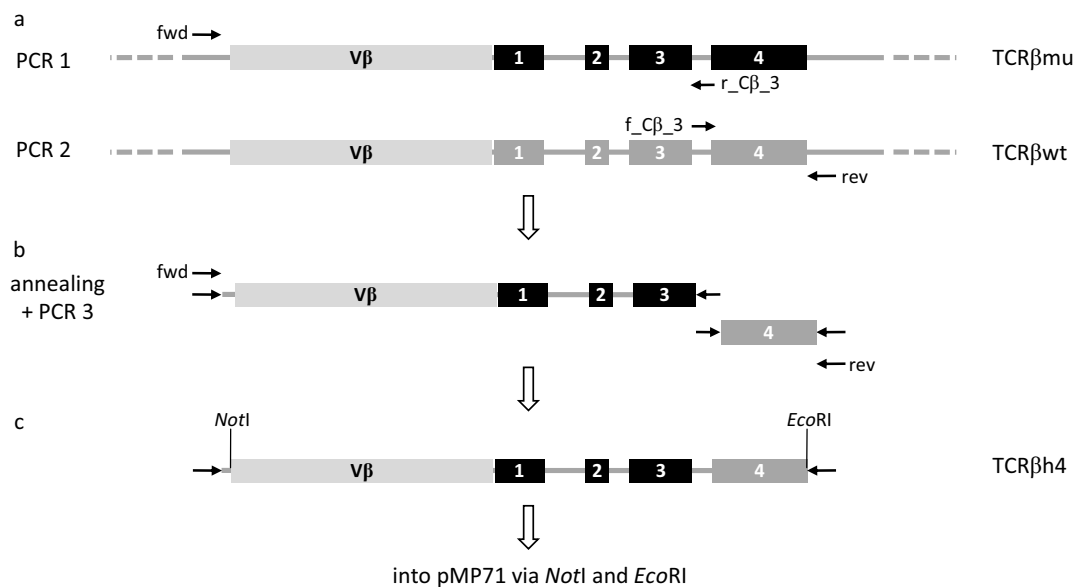


Figure 10: Exchange of C β domain 4 between a human and murine TCR. The construction of the TCR β variant TCR β h4 is shown as an example for the exchange of a domain. (a) One fragment containing the variable region and the murine C β domains 1-3 and one fragment with the human (wild type) C β domain 4 were amplified using the complementary primers f_C β _3 and r_C β _3 and the primers fwd and rev. (b) The two TCR gene fragments were annealed and the complete gene was amplified. (c) TCR β h4 was cloned into pMP71 via *NotI* and *EcoRI* restriction sites.

For cloning of some constructs both strategies (insertion of mutations and combination of different gene fragments) were combined (e.g. for the murinization of TCR C-regions).

Codon-optimization and synthesis of the optimized genes was performed by GENEART (Regensburg, Germany). Genes were cut out from delivered plasmids and cloned into pMP71 via restriction sites *NotI* and *EcoRI*.

3.2.3 Transfection by calcium phosphate precipitation

8×10^5 cells per well were seeded into 6-well-plates in 3 ml medium. One day later 18 μ g DNA were diluted with H₂O to a final volume of 135 μ l and 15 μ l 2.5 M CaCl₂ were added. Then 150 μ l transfection buffer (1% HEPES, 1.5 mM Na₂HPO₄, 270 mM NaCl, 10 mM KCl, pH 6.75) were added drop by drop while vortexing the tube. After 15 min of incubation, the mixture was pipetted in drops to the cells. After 6 h the medium was replaced by 3 ml fresh medium.

3.2.4 Production of virus supernatant

To obtain packaging cells, which stably produce viral particles, retroviral vector plasmids were cotransfected with the plasmid pWLneo (contains a neomycin/geneticin resistance gen, Stratagene, Heidelberg, Germany) using calcium phosphate precipitation into GP+E86 cells to generate ecotropic vector particles or 293-10A1 cells to generate amphotropic vector particles. Transfected clones were isolated by Geneticin (G418, Gibco) selection (1.5 mg/ml). Supernatants of different clones were analyzed for transduction efficiency and clones resulting in high transduction levels were chosen for virus production. Virus supernatant was produced at 37°C, filtered through a 0.45- μ m pore size filter to remove cells and debris, and stored in aliquots at -80°C.

To obtain cells transiently producing amphotropic retroviral particles, 293T cells were transfected with the respective TCR encoding retroviral vector plasmids and expression plasmids encoding the Moloney MLV gag/pol gene (pcDNA3.1MLVg/p, kindly provided by C. Baum, Hannover, Germany) and the MLV-10A1 env gene (pALF-10A1, (Stitz *et al.*, 2000)). 293T cells were transfected with a total amount of 18 μ g DNA (TCR α : 3.5 μ g, TCR β : 3.5 μ g, gag/pol: 6 μ g, env: 5 μ g for single chain vectors or TCR α/β : 6 μ g, gag/pol: 6 μ g, env: 6 μ g for P2A-vectors) by calcium phosphate precipitation. 48 h after transfection, viral supernatant was filtrated and used directly for transduction. If supernatant for a second transduction on the following day was needed, 3 ml fresh medium were added to the cells after harvesting the supernatant.

For transient production of ecotropic retroviral particles, Plat-E cells were transfected with 18 μ g of the respective TCR encoding retroviral vector plasmid.

3.2.5 Transduction of T cells

T cells (T cell lines: 1.5×10^5 or human CTL (T21 and InRi1): 5×10^5 cells per well) were transduced in 24-well non-tissue culture plates coated with RetroNectin (3.5 μ g/well) (Takara, Apen, Germany) with 1 ml retrovirus supernatant supplemented with protamine sulfate (final concentration 4 μ g/ml, Sigma-Aldrich). After addition of supernatant, plates were spinoculated with 800 g for 1.5 h at 32°C. CTL were transduced three days after restimulation with anti-CD3 mAb.

Human PBL were transduced at day two after isolation by adding 1 ml viral supernatant supplemented with protamine sulfate and IL-2 (100 U/ml) to the stimulated cells. Plates were spinoculated as described above. Transduction of PBL was performed for a second time on the subsequent day on RetroNectin coated 6-well plates, which were preloaded with virus for 1 h at 4°C. 3 ml medium containing 100 U/ml IL-2 were added. On day five or six after isolation, PBL were transferred into cell culture flasks and cultured with 100 U/ml IL-2 until day thirteen. Then, cells were rested by reducing the IL-2 concentration to 10 U/ml IL-2.

3.2.6 Flow cytometry

In a flow cytometer cells are separated and single cells flow through a capillary. There they pass light beams with different wave lengths and intensity of emitted light is measured.

Approximately 5×10^5 cells were labeled using mAb or MHC-multimers in PBS. After 20-90 min cells were washed with PBS and fluorescence intensity was measured using a FACSCalibur flow cytometer (BD) and Cellquest Pro software (BD). Data were analyzed using FlowJo software (Tree Star, Ashland, USA).

3.2.7 Fluorescence activated cell sorting (FACS)

FACS is based on flow cytometry, but in addition cells with desired properties can be collected. Approximately 1×10^7 gp100-TCR transduced human CTL T21 and InRi1 were labeled with reversible gp100-multimers and sorted with a Moflo cell sorter (Cytomation, Fort Collins, USA).

3.2.8 Magnetic cell sorting (MACS)

For MACS, cells are labeled with magnetic beads, which enables the separation of labeled and unlabeled cells in a magnetic field.

Approximately 1×10^7 P14 α , P14 β , and P14 α/β transduced B3Z cells were incubated with PE-labeled anti-mV α 2 and anti-mV β 8 mAb, respectively. After washing 30 μ l bead-coupled anti-PE Ab (anti-PE-MultiSort-beads, Miltenyi Biotec, Bergisch Gladbach, Germany) were added. Cells were incubated for 15 min at 4°C and afterwards washed. Labeled cells were positively sorted with an autoMACS (Miltenyi Biotec).

3.2.9 Measurement of intracellular β -galactosidase activity

4×10^5 B3Z cells were cocultured with 4×10^5 irradiated (24 Gy) splenocytes from C57BL/6 mice (Jackson, Bar Harbor, USA) and 1 μ M peptide over night in 24-well-plates. After fixation (0.05% glutaraldehyde), X-gal-solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 1 mM MgCl₂ and 2.5 M 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)) was added and cells were incubated for 24 h at 37°C.

3.2.10 Cytokine release assay

Untransduced or TCR modified PBL (1×10^5 per well) were cocultured with 5×10^4 target cells in 200 μ l medium in round bottom 96-well-plates on day fifteen after isolation. As target cells either tumor cell lines or peptide pulsed T2 cells were used. For peptide loading, T2 cells were incubated with different peptide concentrations (e.g. 1 μ M, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM) in serum-free medium for 2 h at 37°C. Then, cells were washed, resuspended in fresh medium and distributed into 96-well-plates together with the PBL. Supernatants obtained after 24 h were analyzed for human IFN- γ content by enzyme linked immunosorbent assay (ELISA, BD Biosciences) in MaxiSorp-96-well-plates (Nunc, Langenselbold, Germany). ELISA were performed according to the manufacturer's protocol with small differences: all antibodies were utilized in a four times lower concentration and for each step only half of the suggested volume was used. IFN- γ concentrations are given as mean values of duplicates with mean deviation.

Additional cytokines were measured using a Cytometric Bead Array (Human Th1/Th2 Cytokine Kit, BD Bioscience) according to the manufacturer's protocol but using only one fifth of the recommended volumes. Data were acquired using a FACSCalibur flow cytometer and Cellquest Pro software and analyzed using FCAP Array Software (BD). With the Cytometric Bead Array the concentration of different cytokines can be measured simultaneously. Ab directed against each cytokine are linked to beads with different fluorescence intensities. After binding of cytokines to the beads, Ab detecting the cytokines are added. These Ab are marked with a second fluorochrome. After measurement with a flow cytometry, cytokines can be distinguished by the intensity of the first fluorochrome and concentrations can be calculated by the intensities of the second fluorochrome.

3.2.11 Cytotoxicity assay

Cytolytic activity was analyzed in a standard 4 h chromium release assay. 5×10^5 T2 cells were labeled with 100 μ Ci ^{51}Cr (ICN Biochemicals, Irvine, USA) in 100 μ l FCS and then loaded with 10 μ g/ml peptide for 1 h at room temperature. T cells were serially diluted, resulting in graded effector to target (E:T) ratios. ^{51}Cr -labeled target cells were cocultured with T cells in 200 μ l medium per well in V-bottom 96-well tissue culture plates. After 4 h at 37°C, 100 μ l of each supernatant was collected and radioactivity was analyzed in a scintillation counter. Cytolytic activity was calculated as the percentage of specific ^{51}Cr release using the following equation: % specific lysis = $100 \times (\text{sample release} - \text{spontaneous release}) : (\text{maximal release} - \text{spontaneous release})$. Data refer to mean values of duplicates with mean deviation.

4 Results

4.1 The concept of strong and weak TCR

As described in chapter 1.3, after TCR gene transfer into T cell theoretically at least four different TCR α / β combinations can be expressed, the endogenous, the transferred and two mixed TCR. As it was not clear, which TCR α / β combinations are really expressed, the influence on the endogenous TCR after TCR gene transfer into T cells was analyzed in different murine and human models.

4.1.1 *Expression of a second TCR replaces the endogenous TCR on a murine T cell line*

The murine T cell line B3Z expresses OVA specific TCR (mV α 13, mV β 5). Additionally, β -galactosidase is expressed under the control of the minimal human IL-2 promoter as a reporter gene for MHC-peptide recognition. B3Z cells were transduced with the LCMV-gp₃₃ specific P14-TCR (mV α 2, mV β 8) and analyzed by flow cytometry after enrichment for transduced cells. Almost 100% of the cells were mV α 2- and mV β 8-positive (Figure 11a) and bound the P14 specific MHC-tetramer (Figure 11b) while untransduced cells were mV α 2-, mV β 8-, and P14-tetramer-negative. Surprisingly, staining of P14 transduced B3Z cells with anti-mV β 5 (B3Z) and anti-mV β 8 (P14) mAb showed no mV β 5 expression on the cell surface, whereas mV β 8 was strongly expressed (Figure 11c). The absence of the endogenous B3Z-TCR (mV β 5) chain on the surface of B3Z-P14 cells was not caused by the MACS enrichment and a selection for cells expressing high amounts of P14-TCR. In a population of partially transduced, unsorted cells, the transduced cells also almost completely downregulated the endogenous B3Z-TCR (mV β 5) only one week after transduction (Figure 11d). The endogenous B3Z TCR α chain (mV α 13) could not be analyzed due to the lack of anti-mV α 13 Ab. The loss of expression of the endogenous TCR β chain raised the question of antigen specificity of P14 transduced B3Z cells. B3Z cells were incubated with irradiated splenocytes loaded with SIY, ova₂₅₇ or gp₃₃ peptide. The control peptide SIY did not induce any β -galactosidase activity (Figure 11e). Stimulation with gp₃₃ peptide induced strong β -galactosidase expression in P14 transduced B3Z but not in untransduced B3Z cells, demonstrating that the transduced cells had acquired new antigen specificity. Conversely, incubation with the ova₂₅₇ peptide resulted in β -galactosidase expression in B3Z cells; whereas P14 transduced B3Z cells had almost completely lost OVA antigen specificity. This result demonstrates that TCR gene transfer into a T cell not only grafts it with a new antigen specificity, but it can also completely eliminate the original antigen specificity. This clinically ideal situation can best be explained by the assumption that some TCR are strong and others are weak in terms of cell surface expression under competitive conditions.

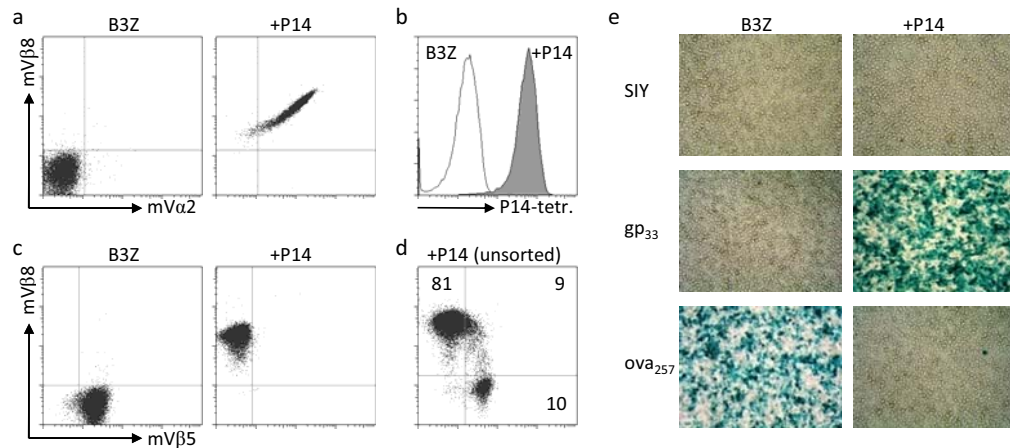


Figure 11: Exchange of antigen specificity by gene transfer in mouse T cells. The OVA specific T cell line B3Z (mV α 13, mV β 5) was transduced with the LCMV-gp₃₃ specific P14-TCR (mV α 2, mV β 8). Enriched cells were analyzed for (a) mV α 2 and mV β 8 expression and (b) P14-tetramer binding by flow cytometry. (c) Endogenous mV β 5 and exogenous mV β 8 expression was analyzed as indicated. (d) To exclude that loss of mV β 5 expression in P14 transduced B3Z cells was due to cell sorting, unsorted cells one week after transduction are shown. Numbers indicate percentage of positive cells. (e) P14 transduced B3Z cells are specific for gp₃₃ and lost OVA specificity. Untransduced or P14 transduced B3Z cells were incubated with 1 μ g/ml peptide (SIY, gp₃₃ or ova₂₅₇) loaded splenocytes as indicated. After 12 h, β -galactosidase expression, which is induced in B3Z cells after MHC-peptide recognition, was analyzed by X-gal staining.

4.1.2 Expression of a second TCR replaces the endogenous TCR on human T cells

We transduced different human T cells with a human TCR to determine whether similar exchanges of TCR can be found in the more relevant human situation. First, the CMV specific CTL line T21 (V β 20) was stimulated with anti-CD3 mAb and after three days transduced with a gp100 specific TCR (gp100-TCR, V α 19, V β 8). Cells were analyzed with gp100- and CMV-tetramers fourteen days after transduction. On T21 cells expressing the gp100-TCR (14%) the CMV-TCR was no longer detectable on the cell surface (Figure 12a). T21 cells expressing gp100-TCR were enriched by FACS with reversible gp100-multimers, expanded for two weeks, and then expression of endogenous and transduced TCR was again analyzed. Untransduced cells were only positive for the CMV-tetramer (96%) (Figure 12b), while most of the gp100-TCR transduced T21 cells were negative (84%), demonstrating that downmodulation of the endogenous TCR through the exogenous one can also occur on human T cells. To examine function and specificity of the transduced T cells, a cytotoxicity assay was performed with peptide loaded T2 cells two weeks after sorting. Cytotoxic activity of gp100-TCR transduced T21 cells against gp100 peptide loaded T2 cells showed that those cells were equipped with a new specificity (Figure 12c). The cytotoxic activity of gp100-TCR transduced T21 cells against CMV peptide loaded T2 cells was clearly reduced, albeit not completely inhibited, compared to untransduced T21 cells. Neither transduced nor untransduced cells showed reactivity against control peptide (HIV) loaded cells. The residual CTL activity of gp100-TCR transduced T21 cells against

CMV peptide loaded cells was possibly due to the 4% untransduced T cells remaining after sorting, because a small number of these T cells are able to mediate a high lysis due to the expression of a high affinity CMV specific TCR. In addition, sufficient CMV specific TCR molecules might have persisted on the surface of gp100-TCR expressing cells allowing recognition of T2 cells loaded with excess amounts of CMV peptide.

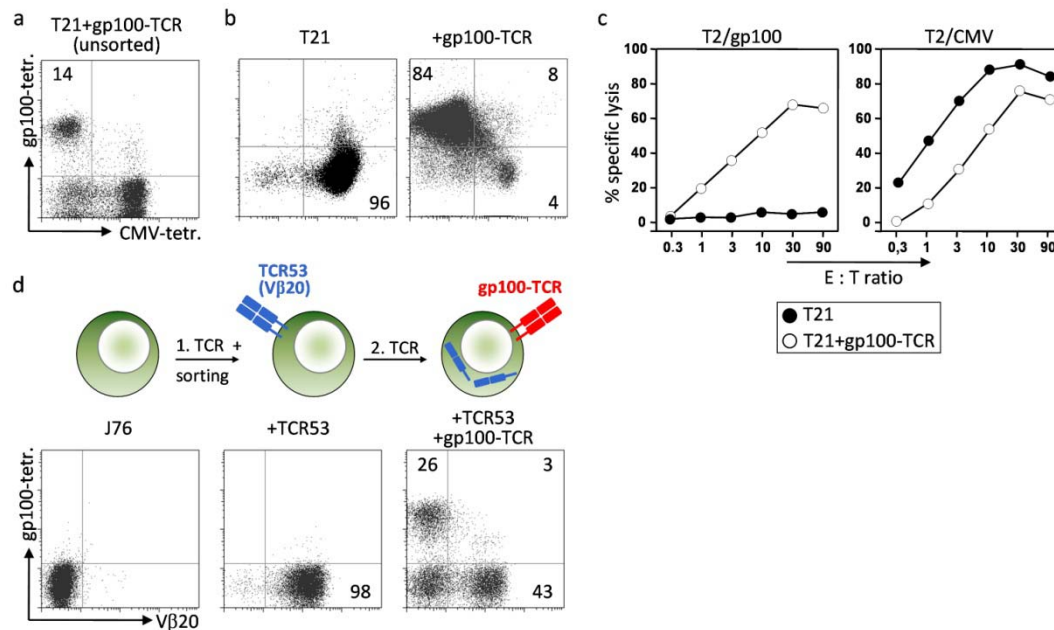


Figure 12: Partial exchange of antigen specificity by gene transfer in human T cells. (a) The CMV specific CTL line T21 (Vβ20) was transduced with a gp100 specific TCR (Vα19, Vβ8) and after fourteen days analyzed for CMV- and gp100-TCR expression using specific MHC-tetramers. (b) Transduced T21 cells were enriched with reversible gp100-multimers, expanded and analyzed again by flow cytometry. (c) A cytotoxicity assay was performed with untransduced and transduced cells. T2 cells loaded with CMV or gp100 peptide (10 μg/ml) were used as targets and E:T ratios from 0.3 to 90 were tested. (d) The TCR deficient human T cell line J76 was transduced with the RCC reactive TCR53 (Vα19, Vβ20) and enriched for TCR expressing cells. Then J76/TCR53 cells were transduced with the gp100-TCR as a second TCR. Five days after transduction, surface expression of both TCR was analyzed after staining with gp100-tetramers and anti-Vβ20 mAb. Percentages of positive cells are indicated.

In the previous example, expression of the exogenous TCR was driven from the retroviral LTR promoter and the endogenous TCR was expressed by the cellular promoter. Therefore, it could not be excluded that different promoter strengths contributed to the different surface expression levels. To express both TCR under control of the same promoter, the TCRα/β deficient human T cell line J76 was transduced first with the RCC reactive TCR53 (Vα19, Vβ20), sorted for TCR expressing cells (98%) and then transduced with the gp100-TCR as a second TCR (Figure 12d). In this case, both TCR were expressed by the same vector (MP71). After five days, cells were stained with Vβ20 specific mAb and gp100-tetramers showing 26% gp100-tetramer-positive cells. As observed in the example above, these cells could no longer be stained with Vβ20 specific mAb, indicating that the strong gp100-TCR

not only downregulated the weak CMV specific T21-TCR but also the weak TCR53. The gp100-tetramer-negative, V β 20-negative J76 cells were most likely transduced with the gp100-TCR β chain but not with the gp100-TCR α chain. In conclusion, the expression of several combinations of two human TCR on one T cell allowed us to identify strong and weak TCR. This property is inherent to the TCR sequence and not due to different promoter usage.

4.1.3 Two strong TCR are coexpressed on human cells

As a third model with human cells, we used the Melan-A specific T cell clone InRi1 (V α 2, V β 14). Cells were transduced with the gp100-TCR and enriched for transduced cells. Unlike what we observed in the first example, most of the gp100-TCR transduced cells coexpressed the Melan-A- and the gp100-TCR (18% for unsorted and 80% for sorted cells), even though Melan-A-tetramer staining was reduced on gp100-TCR expressing cells (Figure 13a/b). Untransduced and gp100-TCR transduced InRi1 cells were analyzed in a cytotoxicity assay. As expected, gp100 peptide loaded T2 cells were only killed by gp100-TCR transduced cells (Figure 13c). Cytotoxic activity of gp100-TCR transduced InRi1 cells against Melan-A peptide loaded T2 cells was not altered in comparison to untransduced cells. No cytotoxicity was found against control peptide (HIV) loaded T2 cells.

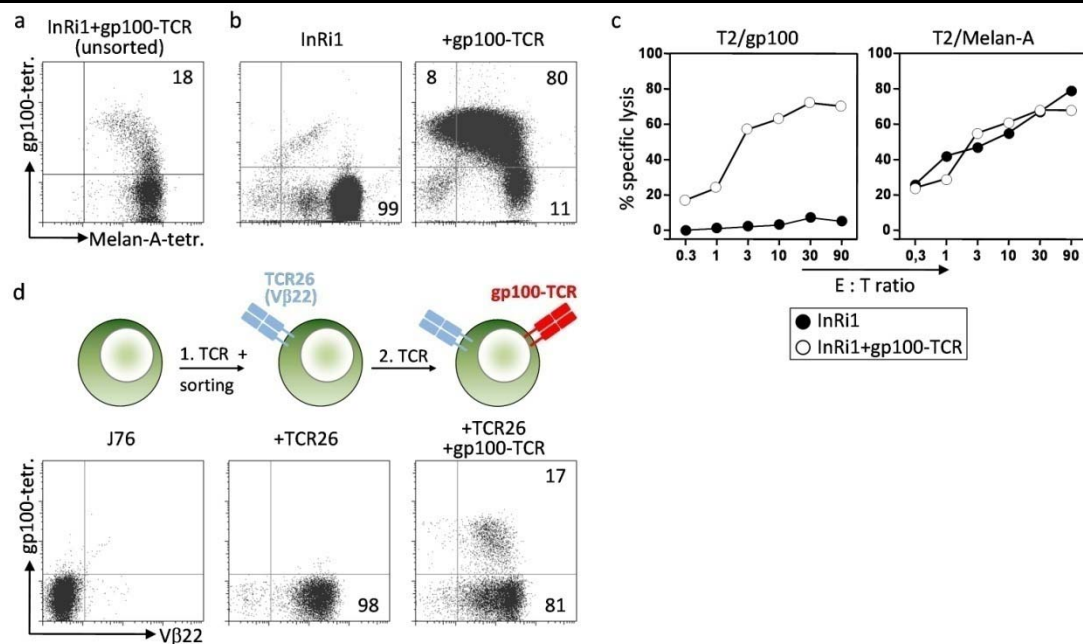


Figure 13: Coexpression of two strong TCR. (a) The Melan-A specific CTL clone InRi1 (V β 14) was transduced with a gp100 specific TCR (V α 19, V β 8) and after fourteen days analyzed for Melan-A- and gp100-TCR expression by tetramer staining. (b) Cells were sorted for gp100-TCR expressing InRi1 cells using reversible gp100-multimers and analyzed again. (c) Cytolytic activity against Melan-A or gp100 peptide loaded T2 cells was measured. (d) The TCR deficient human T cell line J76 was transduced with the RCC reactive TCR26 (V α 20, V β 22) and positively sorted for transduced cells. Afterwards, J76/TCR26 cells were transduced with the gp100-TCR as a second TCR. Surface expression of both TCR was analyzed with gp100-tetramers and anti-V β 22 mAb five days later. Percentages of positive cells are indicated.

As a further example and to exclude experimental artifacts of different promoter usage, J76 cells were transduced with the RCC reactive TCR26. As before, enriched J76/TCR26 cells (98%) were transduced with the gp100-TCR as a second TCR (Figure 13d). This resulted in 17% transduced cells that expressed both the TCR26 and the gp100-TCR. The gp100-tetramer-negative J76/TCR26 population with reduced V β 22 expression most likely was transduced with the gp100-TCR β chain but not with the gp100-TCR α chain. The data demonstrate with two different TCR combinations that two strong TCR can be coexpressed on T cells with retention of both antigen specificities.

4.1.4 Mixed TCR are expressed on human T cells

In the previous examples we could show, that not all possible TCR α/β combinations were expressed on the cell surface, but that some combinations (strong TCR) were expressed better than others (weak TCR). However, we had focused on the original TCR α/β combinations and could not exclude the additional formation of mixed TCR for every situation. Therefore, we next examined if mixed TCR could be expressed on human cells under forced conditions when only one (either a TCR α or a TCR β chain) was transferred, which we had shown in mouse models before (Sommermeyer, 2004). The CMV specific CTL line T21 was transduced with gp100-TCR α or gp100-TCR β . Seven days later, cells were stained with mAb directed against the TCR β chains of the endogenous (V β 20) and exogenous TCR (V β 8). Untransduced cells expressed only V β 20 (Figure 14). As expected, after gp100-TCR α transduction the expression of the endogenous TCR β chain did not change and TCR α expression could not be measured as no anti-V α 19 Ab exists. After transduction with gp100-TCR β retrovirus, mixed TCR could be detected, as a population of 54% was positive for the gp100-TCR β chain (V β 8), which could be expressed on the cell surface only together with the endogenous TCR α chain. The two TCR β chains competed with each other for surface expression, because on gp100-TCR β (V β 8) expressing cells the amount of endogenous TCR β chains (V β 20) is reduced compared to untransduced cells (mean fluorescence intensity (MFI): 27 versus 62). By detecting the transferred gp100-TCR β chain on the CTL line T21, mixed TCR could be also revealed on human cells.

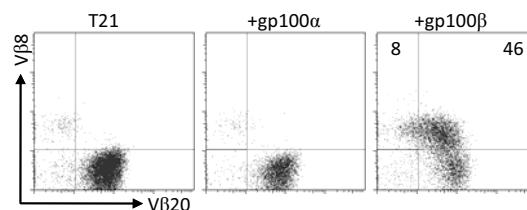


Figure 14: Mixed TCR can be expressed on human cells. The CMV specific CTL line T21 was transduced with gp100-TCR α or gp100-TCR β encoding vectors and seven days later analyzed for expression of T21-TCR β (V β 20) and gp100-TCR β (V β 8) by flow cytometry. Percentages of cells are indicated.

4.1.5 *Trans-species TCR heterodimers of murine and human TCR chains can be expressed*

Next, we asked whether mixed TCR can only be formed by TCR α and TCR β chains of one species, e.g. human or mouse, or if those molecules can be formed by TCR chains of different species. For this purpose, human and murine T cell lines were transduced with single TCR chains of the other species. First, the human T cell line HuT78 was transduced with P14 α , P14 β or both retroviruses. After transduction with P14 α retroviruses, no clear population expressing the murine V α 2 chain could be identified (Figure 15a). However, mixed TCR of mouse and human chains could be recognized after transduction with P14 β , as 76% of the cells were positive for the P14 β chain (mV β 8). After incubation with both supernatants only P14 β (12%) and double (56%) transduced, but no single P14 α transduced cells could be detected. This indicates that HuT78 could be transduced with the P14 α chain, but this chain could only be expressed together with the P14 β chain. On cells expressing both P14 chains, the expression level of P14 β chains was higher than on cells expressing only the P14 β chain, indicating that the pairing of the murine P14 β chain was more efficient with the murine P14 α chain than with the human endogenous chain.

Second, the mouse T cell line B3Z was transduced with the human gp100-TCR β chain. After five days, cells were stained with V β 8 mAb. 47% of the cells exposed to the gp100-TCR β retrovirus expressed the human V β 8 chain (Figure 15b), demonstrating that mixed TCR of gp100-TCR β and the endogenous murine TCR α chain could reach the surface of B3Z cells. However, the expression of the gp100-TCR β chain was low, indicating that only few of those TCR β chains were expressed.

These two examples showed that the formation of mixed TCR consisting of one human and one murine TCR chain was possible, but less efficient compared to the formation of TCR consisting of chains from one species.

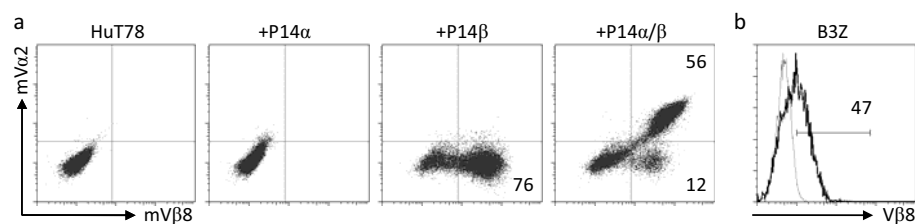


Figure 15: Mixed human/mouse TCR can be expressed on human and murine T cell lines. (a) The human T cell line HuT78 was transduced with P14 α , with P14 β and with both retroviruses and after three days analyzed for surface expression of mV α 2 and mV β 8. (b) The murine T cell line B3Z was transduced either with P14 β (gray line) or with gp100-TCR β (black line). Five days later, cells were stained with anti-human-V β 8 mAb. Percentages of cells are indicated.

4.1.6 A murine TCR is stronger than human TCR on human cells

Finally, we asked whether murine TCR can be efficiently expressed on human T cells and even downregulate human TCR. Therefore, J76/TCR26 and J76/TCR53 cells (Figure 12d and Figure 13d) were transduced with the P14-TCR. In this experiment, a mouse TCR was expressed on cells with a single human TCR so that their reciprocal influence on expression could be analyzed. 51% of J76/TCR26 cells and 54% of J76/TCR53 cells were transduced with the P14-TCR (Figure 16). Remarkably, in both cell lines the human TCR cell surface expression was nearly completely downregulated by the murine TCR, as shown by human $\text{V}\beta 20$ - and $\text{V}\beta 22$ -staining, respectively. These results indicate that murine TCR are stronger than human ones.

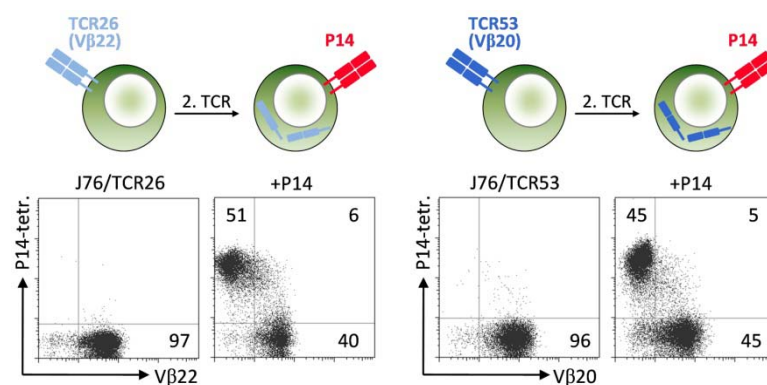


Figure 16: Human TCR replacement by a murine TCR on human T cells. J76/TCR26 and J76/TCR53 cells (as shown in Figure 12d and Figure 13d) were transduced with the murine P14-TCR. Five days after transduction, TCR expression was visualized by staining with P14-tetramers and with anti- $\text{V}\beta 20$ and anti- $\text{V}\beta 22$ mAb, respectively. Percentages of cells are indicated.

4.2 Comparison of strategies to improve the functional expression of therapeutic TCR

As shown in chapter 4.1, different $\text{TCR}\alpha/\beta$ combinations are differently well expressed on the cell surface, which is likely due to unequal stabilities within the TCR complex. Only a strong TCR - in terms of cell surface expression - is displayed at a level sufficient to endow T cells with the desired antigen specificity. To further improve the expression of strong TCR and even to enable surface expression of weak TCR, strategies are needed to optimize therapeutic TCR expression (Uckert and Schumacher, 2009).

4.2.1 Codon-optimization of a NY-ESO-1 specific TCR results in increased TCR expression and slightly enhanced functional avidity of transduced T cells

Different strategies for the optimization of therapeutic TCR have been developed (chapter 1.4). For comparison of these strategies we first used a NY-ESO-1 specific TCR (NY-TCR) and cloned the following constructs as $\text{TCR}\beta$ -2A- $\text{TCR}\alpha$ constructs into the retroviral vector MP71: wild type (wt,

unmodified), murinized (mu), with additional disulfide bond (cys), codon-optimized (co), murinized and additional disulfide bond (mucys), additional disulfide bond and codon-optimized (cysco), and murinized and codon-optimized (muco). The different constructs were transduced into J76 cells and human PBL in parallel. Staining of J76 cells with anti-CD3 mAb four days after transduction revealed similar transduction efficiency for all constructs (transduction rate: 80-90%) (Figure 17a).

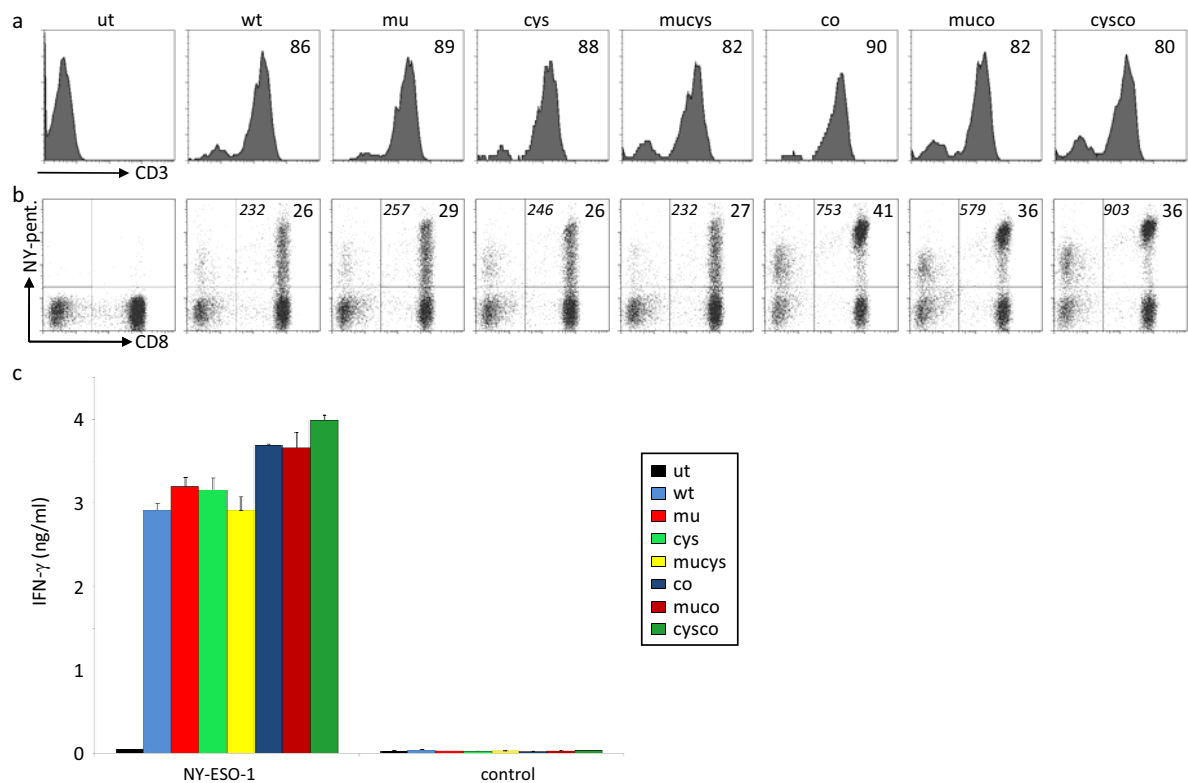


Figure 17: Higher functional avidity with codon-optimized NY-TCR. Different variants (wt, mu, cys, mucys, co, muco, cysco) of NY-TCR were cloned into the retroviral vector MP71 as P2A constructs. J76 (a) and human PBL (b) were transduced and surface expression of NY-TCR was analyzed after staining with anti-CD3 mAb or anti-CD8 mAb and NY-pentamer. MFI (italic) and percentages of CD8/pentamer-positive cells are indicated. (c) PBL were cocultured with T2 cells pulsed with 10 nM NY-ESO-1 or control peptide and IFN- γ concentrations of collected supernatants were analyzed by ELISA.

PBL were stained on day thirteen with anti-CD8 mAb and NY-ESO-1-pentamers. Untransduced (ut) cells did not bind the pentamer, but cells transduced with all different NY-TCR constructs showed pentamer binding for CD8⁻ as well as CD8⁺ cells, indicating that also the NY-TCRwt is functionally expressed (CD8/pentamer-positive: 26%, MFI: 232) (Figure 17b). The modifications murinization, additional disulfide bond or a combination of both resulted in a small or nearly no improvement (CD8/pentamer-positive: 26-29%, MFI: 232-257). However, after codon-optimization (either alone or in combination with other strategies) a substantial increase of pentamer-positive cells (CD8/pentamer-positive: 36-41%) and in the level of expression (MFI: 579-903) was found (Figure 17b). The higher number of pentamer-positive cells is most probably not due to higher transduction

rates as no differences in transduction levels were observed using J76 cells, but due to a better expression of the TCR on transduced cells. To analyze if the improved expression also resulted in an enhanced function of transduced cells, PBL were cocultured with T2 cells pulsed with titrated amounts of NY-ESO-1 peptide and cell culture supernatants collected after 24 h were analyzed for IFN- γ concentrations. The concentrations correlated with expression levels of the NY-TCR (Figure 17c), as all cells transduced with codon-optimized TCR released more IFN- γ than cells transduced with non-codon-optimized TCR genes (Figure 17c shows IFN- γ concentrations for 10 nM peptide). However, the differences in the amount of released IFN- γ were relatively small. Murinization and additional disulfide bond resulted in slightly increased IFN- γ concentrations compared to wild type TCR transduced cells. None of the different cell populations reacted against T2 cells pulsed with a control peptide (Figure 17c). Codon-optimization led to more released IFN- γ but not to higher affinity of the NY-TCR, as for all variants T2 cells pulsed with 0.1 nM were not recognized. This finding was expected, as the modification changed the ability of the TCR to be expressed, but not the antigen binding region of the TCR. Therefore, the functional avidity of the transduced cells was higher, but not the affinity of the NY-TCR.

4.2.2 *Weak TCR need a combination of different optimization strategies for a high functional expression*

For NY-TCR codon-optimization was most efficient, however, none of the tested strategies substantially increased the functional avidity of transduced cells. To analyze if this is generalizable, we used TCR53 as a second example, for which we have shown that it is a weak TCR (chapter 4.1.2).

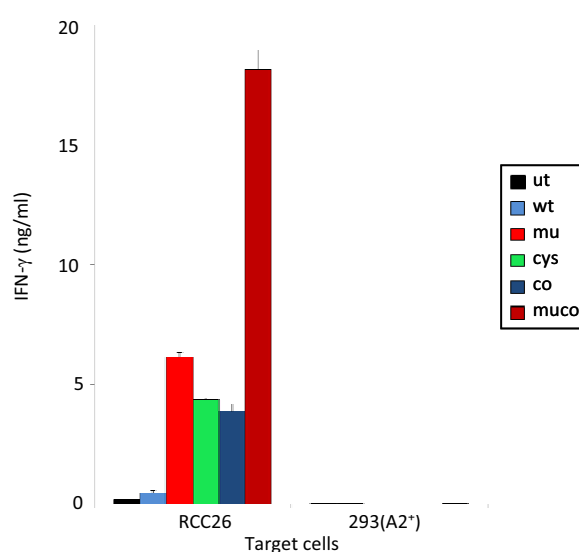


Figure 18: High functional avidity after optimization of a weak TCR. Human PBL were transduced with different variants of TCR53 and cocultured with the antigen positive RCC cell line RCC26 and the antigen negative cell line 293(A2⁺). IFN- γ concentrations of supernatants collected after 24 h were analyzed by ELISA.

Different variants of the TCR (wt, mu, cys, co, muco) were cloned as P2A constructs into the retroviral vector MP71 and transduced into human PBL. As the antigen of this TCR is still unknown, no multimer staining was possible. Therefore, PBL were cocultured with a RCC cell line (RCC26), which had been recognized by the tumor infiltrating lymphocyte clone, from which this TCR had been isolated. TCR53wt transduced cells secreted only low amounts of IFN- γ (0.5 ng/ml) whereas PBL transduced with TCR optimized with one strategy (mu, cys, or co) released substantially more IFN- γ (3.9-6.1 ng/ml) (Figure 18). Most IFN- γ was released by PBL transduced with TCR53muco, reaching nearly 40 times more IFN- γ (18.1 ng/ml) compared to TCR53wt. Antigen negative 293(A2⁺) cells were not recognized. This example showed that the optimization of weak TCR by codon-optimization, murinization, or additional disulfide bond is needed for functional expression on PBL, and that a combination of modifications can further increase the function of transduced cells.

4.2.3 Optimization of different tyrosinase specific TCR enhances the functional avidity of transduced cells

The previous example has shown that a dramatic increase of functional avidity can be reached using the combination of murinization and codon-optimization. Therefore, we used this combination to improve the functional avidity of T cells transduced with different tyrosinase specific TCR (D115, T58, IVS-B). The three TCR originated from different approaches, IVS-B was isolated from a CTL clone of a melanoma patient (Wolfel *et al.*, 1993). D115 and T58 were generated by “reverse immunology”, D115 in an autologous and T58 in an allogeneic approach (Wilde *et al.*, 2009). Wild type and optimized (muco) TCR chains were cloned as P2A constructs into the retroviral vector MP71 and transduced into J76 cells and human PBL. Transduction efficacy of J76 cells was between 77% and 96% (Figure 19a). PBL were analyzed thirteen days after isolation for expression of tyrosinase specific TCR on CD8⁺ cells by staining with tyrosinase-tetramers. For all three analyzed TCR, the optimization (muco) led to a substantial increase of CD8/tetramer-positive cells (D115: 2.4% → 12.2%, T58: 0.7% → 14%, IVS-B: 0.2 → 12.8%) and in level of expression (MFI of CD8/tetramer-positive cells: D115: 112 → 401, T58: 72 → 285, IVS-B: 23 → 244) (Figure 19b). To examine whether the increased number of tetramer-positive cells also led to an enhanced function of transduced cells, PBL were cocultured with T2 cells pulsed with titrated amounts of tyrosinase peptide. PBL transduced with optimized (muco) TCR released significantly more (e.g. 2.7x for D115, 11.5x for T58, and 19.7x for IVS-B at 100 nM peptide) IFN- γ than PBL transduced with unmodified TCR (Figure 19c). PBL transduced with the optimized T58 TCR showed background activity against T2 cells, while all other PBL did not react against T2 cells pulsed with control peptide.

To test reactivity against tumor cells, PBL were cocultured with the melanoma cell lines Mel-624.38 (tyrosinase⁺) and Mel-A375 (tyrosinase⁻). As for T2 cells, PBL transduced with the optimized (muco)

TCR released more IFN- γ (22x for D115 and 79x for T58) compared to cells transduced with the wild type TCR (Figure 19d); for IVS-B TCR the optimization modified a non-reacting TCR into a well reacting one. Cocultivation with the tyrosinase⁻ cell line Mel-A375 led to a little background IFN- γ release for all PBL.

Comparing the three different wild type TCR, the data also indicated that D115 is the strongest of the three TCR in matters of surface expression, as tetramer binding was best for this TCR.

Those three examples of tyrosinase specific TCR showed that using the combination of murinization and codon-optimization the performance of a TCR can be clearly improved.

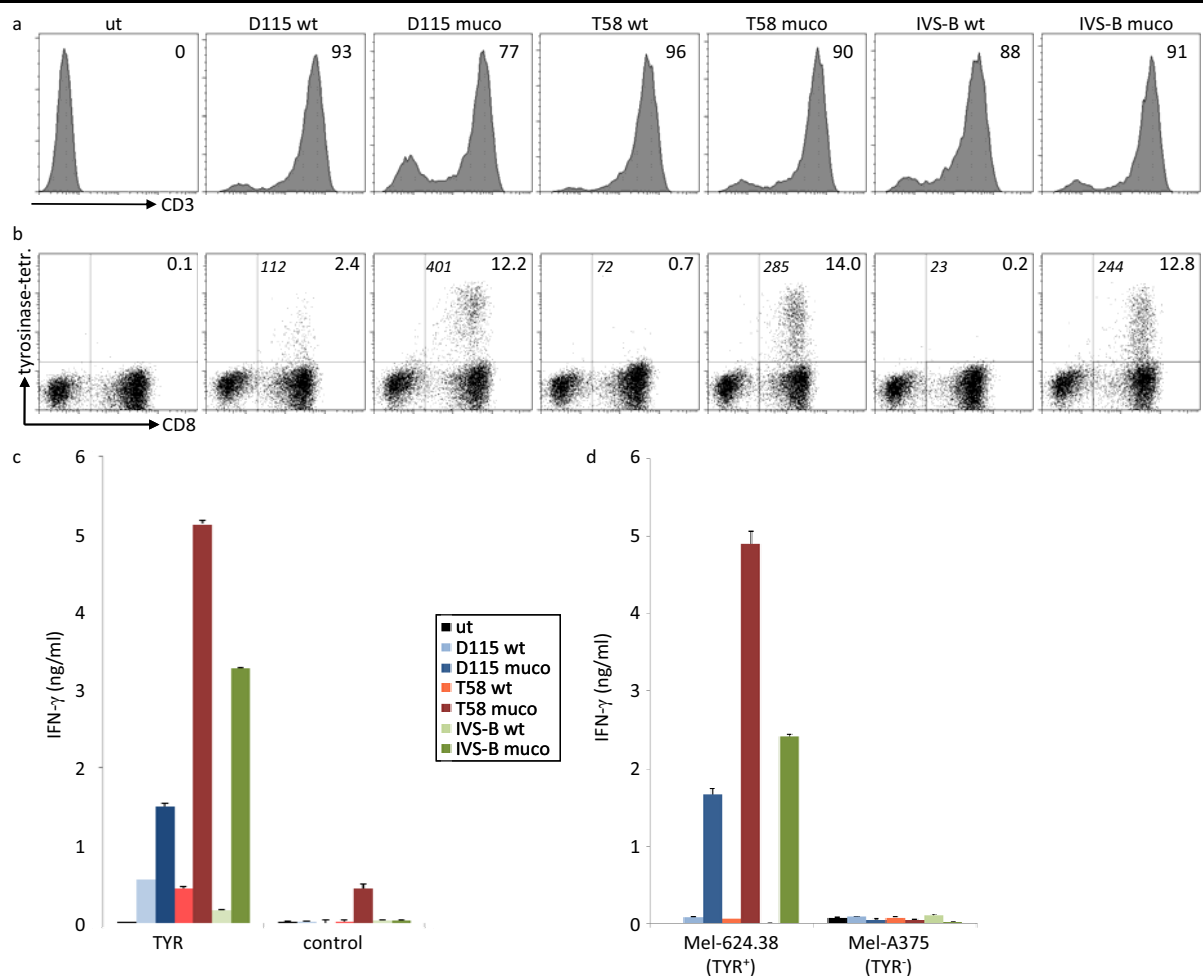


Figure 19: Optimization of tyrosinase specific TCR increased the function of transduced cells. Three tyrosinase specific TCR (D115, T58, and IVS-B) were cloned into the vector MP71 either as wild type (wt) TCR or in an optimized (muco) variant. With these constructs J76 cells (a) and human PBL (b) were transduced and cell surface expression of different TCR was analyzed after staining with anti-CD3 mAb or anti-CD8 mAb and tyrosinase-tetramer. MFI (italic) and percentages of CD8/tetramer-positive cells are indicated. (c,d) PBL were cocultured with T2 cells pulsed with 100 nM tyrosinase or control peptide and melanoma cell lines Mel-624.38 (tyrosinase⁺) or Mel-A375 (tyrosinase⁻), respectively. IFN- γ concentrations of supernatants collected after 24 h were analyzed by ELISA.

4.3 How much “murinization” does a human TCR need for efficient cell surface expression?

4.3.1 Only murinized, but not wild type, NY-TCR can be expressed on J76/TCR26 cells

The improved functional expression of human TCR in which the C-regions were replaced by murine counterparts (murinized TCR) in comparison to wild type TCR was reported (Cohen *et al.*, 2006) and confirmed with examples described in chapter 4.2. For the identification of the amino acids within the C-regions of the TCR α and TCR β chains that are responsible for improved expression of murinized TCR, we established a TCR replacement model. It is based on our observations that certain TCR α/β combinations, when transferred into T cells expressing endogenous TCR, are better expressed (strong TCR) than others (weak TCR) (chapter 4.1). We created a setting where the NY-TCR had to compete with the strong TCR26 (chapter 4.1.3). For this, we used J76/TCR26 cells and transduced them with the NY-TCR, either in a wild type (wt) or murinized (mu) form. Staining with mAb directed against the V-regions of TCR26 β (V β 22) and NY-TCR β (V β 4) allowed expression of both TCR to be determined by flow cytometry.

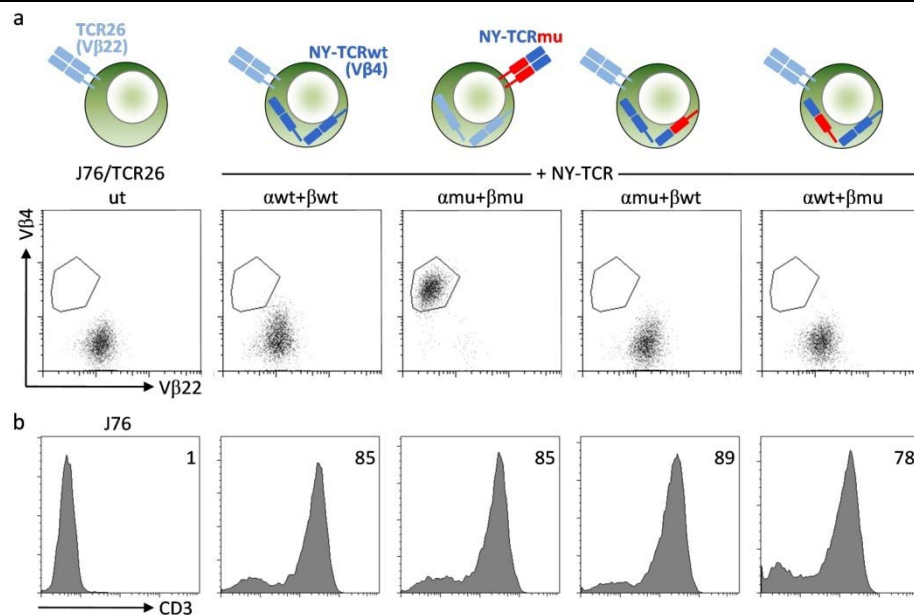


Figure 20: Murinization of NY-TCR enables expression on J76/TCR26 cells. Different combinations of wild type (wt) and murinized (mu) TCR α and TCR β chains of NY-TCR were transduced into (a) J76/TCR26 and (b) J76 cells. Cells were stained for the expression of V β 4 (NY-TCR), V β 22 (TCR26) and CD3, respectively, four days after transduction. Untransduced (ut) cells were used as control. Percentages of TCR-positive J76 cells are indicated (b).

J76/TCR26 cells transduced with the NY-TCRwt (α wt+ β wt) showed no or only poor expression of NY-TCR while the expression of TCR26 remained unchanged compared to untransduced cells (Figure 20a). In contrast, transduction of J76/TCR26 cells with the NY-TCRmu (α mu+ β mu) revealed cells

expressing NY-TCR and, in addition, the expression of TCR26 was reduced on this population. These results indicated that the murinized variant but not wild type NY-TCR could be expressed on J76/TCR26 cells. Gene transfer of combinations of one murinized and one wild type NY-TCR chain ($\alpha\mu + \beta\text{wt}$ and $\alpha\text{wt} + \beta\mu$, respectively) did not result in the expression of the NY-TCR, demonstrating that the murinization of both chains was decisive for cell surface expression of NY-TCR on J76/TCR26 cells.

To ensure that the expression level of NY-TCR was not due to differences in transduction, we transduced TCR deficient J76 cells in parallel. Staining of these cells using anti-CD3 mAb revealed transduction efficiencies between 78% and 89% (Figure 20b).

4.3.2 Improved expression of murinized TCR is mainly due to a single exchange from an acidic to a basic amino acid within the TCR β chain

For the identification of the amino acids responsible for improved surface expression of murinized TCR, we first focused on the TCR β chain. We defined four domains, which included all differences between the human and mouse sequences, which were flanked by homologous regions (Figure 21a). We constructed four NY-TCR β chains with different C-regions, each containing one human and three murine domains (Figure 21b). The different NY-TCR β constructs were transduced together with the completely murinized NY-TCR α chain gene into J76/TCR26 cells. Four days after transduction, cells were stained with anti-V β 4 and -V β 22 mAb and analyzed by flow cytometry (Figure 22a, Table 5).

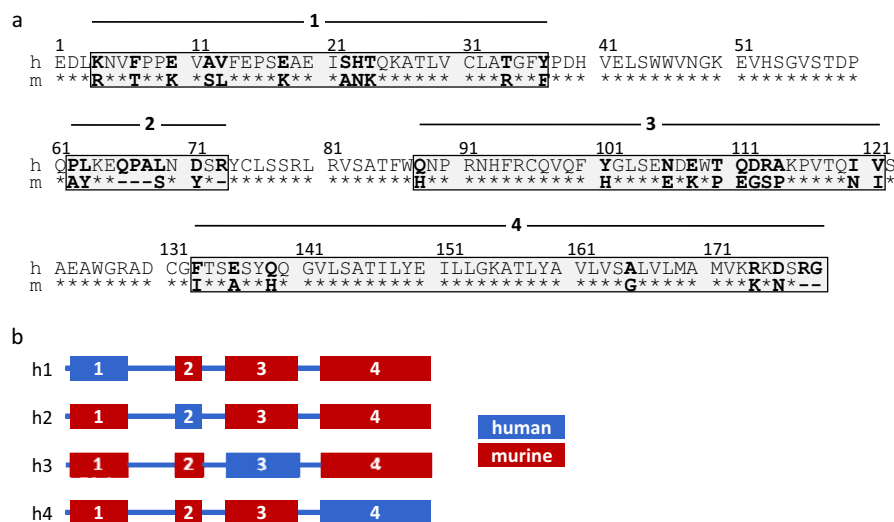


Figure 21: Comparison of human and murine TCR β 2-region reveals clustered differences flanked by homologous regions. (a) The amino acid sequences of human (h) and murine (m) TCR β 2-region were compared and four domains covering all differences were defined (gray boxes). Asterisks represent the same amino acid in the murine sequence. (b) Schematic drawing of four different TCR β C-regions consisting each of one human and three murine domains.

Cells transduced with the construct including the human domain 1 (h1) showed no NY-TCR (V β 4) expression. In contrast, cells transduced with constructs including the human domains 2 (h2) or 3 (h3) demonstrated NY-TCR expression and TCR26 replacement comparable to the completely murinized NY-TCR β chain (Figure 22a/d, Table 5). Transduction with the construct containing the human domain 4 (h4) resulted in a population with lower NY-TCR expression and minor reduction of TCR26 expression. These results suggested that domains 1 and 4 of the mouse C-region were important for the expression of the NY-TCR on J76/TCR26 cells, while domains 2 and 3 were not essential. Moreover, the first domain seemed to be indispensable, because no NY-TCR expression was detected if this part was not murinized. Domain 4 seemed to be less important, but was still needed to increase TCR expression. Consequently, we generated a NY-TCR β chain construct consisting of domain 1 and 4 of the mouse C-region and 2 and 3 of human origin (m1/4). After transduction of this NY-TCR β chain gene together with the murinized NY-TCR α chain gene into J76/TCR26 cells, the NY-TCR expression was comparable to the completely murinized constructs (Figure 22a/d). This result confirmed that the amino acids which are important for the improved expression of the murinized NY-TCR were localized within domains 1 and 4 of the mouse C-region.

Table 5: Summary of the fraction of murinization linked to the ability to replace TCR26.

TCR α + TCR β constructs	Murinization		TCR26 replacement (%)
	C α	C β	
α wt+ β wt	0	0	5
α mu+ β mu	100	100	100
α mu+ β wt	100	0	0
α wt+ β mu	0	100	0
α mu+ β h1	100	71	0
α mu+ β h2	100	79	92
α mu+ β h3	100	71	93
α mu+ β h4	100	79	65
α mu+ β m1/4	100	50	90
α mu+ β m1.2/4.1	100	18	84
α mu+ β m1.2KE/4.1	100	16	25
α mu+ β m1.2AS/4.1	100	16	76
α mu+ β m1.2NH/4.1	100	16	95
α mu+ β m1.2KT/4.1	100	16	87
α mu+ β mm	100	13	87
α mu+ β mmKR	100	13*	90
α m2+ β mm	54	13	86
α mm+ β mm	8	13	83

*The TCR β C-region contains an amino acid (arginine at position 18) which is neither of human nor murine origin.

Next, we subdivided domains 1 and 4 into smaller parts (part 1.1 included amino acids 4-13; part 1.2: 18-24; part 1.3: 34-37; part 4.1: 133-139; part 4.2: 165-179) and replaced each part by the corresponding human sequence. Again, these five constructs were transduced together with the completely murinized NY-TCR α chain gene into J76/TCR26 cells. Flow cytometric analysis revealed that the “murine” amino acids of part 1.1, 1.3, and 4.2 were not required for an efficient expression of NY-TCR. This result was confirmed by the construction of a NY-TCR β chain gene in which only parts 1.2 and 4.1 of the murine sequence were combined (m1.2/4.1) (Figure 22b). In this construct, the number of “murine” amino acids was decreased from 38 to 7 compared to the complete murine C β 2 sequence and the remaining mouse amino acids were K-18, A-22, N-23, K-24, I-133, A-136, and H-139.

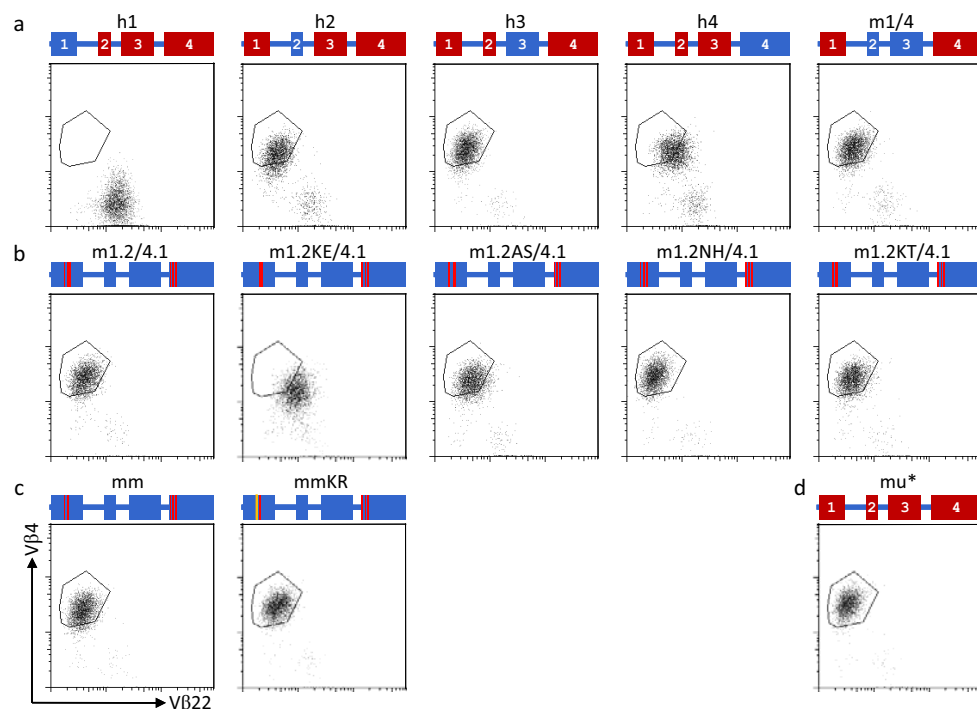


Figure 22: Enhanced TCR expression after murinization can be maintained with only five “murine” amino acids of the TCR β C-region. (a-d) J76/TCR26 cells were transduced with the completely murinized NY-TCR α chain in combination with NY-TCR β constructs containing different C-regions and analyzed for surface expression of TCR26 (V β 22) and NY-TCR (V β 4). *This picture was taken from Figure 20a for comparison.

We continued by mutating each of these seven amino acids of the mouse sequence back to the human sequence. The mutation of the “murine” basic lysine (K-18) to the “human” acidic glutamic acid (E-18) (m1.2KE/4.1) showed the most striking effect, as NY-TCR expression and TCR26 replacement were clearly reduced (Figure 22b, Table 5). The mutation of alanine (A-22) to serine (S-22) (m1.2AS/4.1) caused a smaller effect, while the mutations of asparagine (N-23) to histidine (H-23) (m1.2NH/4.1) and lysine (K-24) to threonine (T-24) (m1.2KT/4.1) had only negligible effects (Figure

22b). For part 4.1 mutations from isoleucine (I-133), alanine (A-136), and histidine (H-139) to the original human residues phenylalanine (F-133), glutamic acid (E-136), and glutamine (Q-139), respectively, yielded a minor decrease of NY-TCR expression for each of the three amino acids.

Finally, a NY-TCR β chain construct was created containing the amino acids K-18, A-22, I-133, A-136, and H-139 of the murine protein. This construct still allowed NY-TCR expression and TCR26 replacement comparable to the completely murinized NY-TCR β chain and was defined as the minimally murinized (mm) TCR β chain construct (Figure 22c/d, Table 5). To analyze whether substitution of other basic amino acids at position 18 would lead to enhanced TCR expression, we mutated lysine to arginine (mmKR) and found a similarly enhanced expression of NY-TCR on J76/TCR26 cells (Figure 22c).

In summary, these studies allowed us to reduce the number of amino acid exchanges from 38 (completely murinized) to 5 (minimally murinized) on the TCR β chain while maintaining nearly the same enhanced level of expression of NY-TCR. The most important difference between the human and murine C β 2-regions was identified at position 18, where the murine sequence contained the basic amino acid lysine, while the human sequence included the acidic amino acid glutamic acid. The procedures used to identify the relevant positions in the TCR β C-region are summarized in Figure 23.

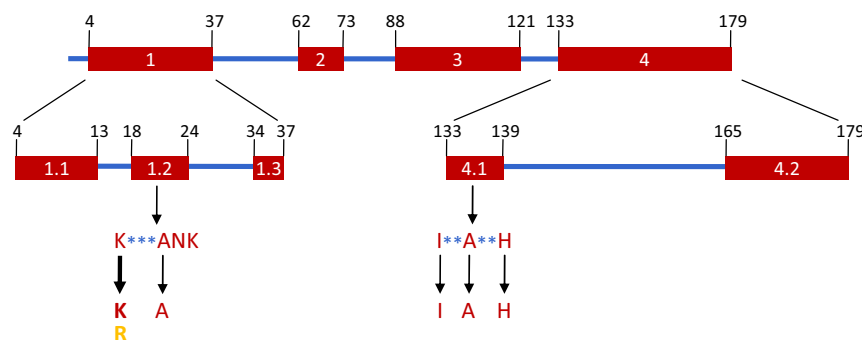


Figure 23: Schematic summary of the procedures to identify the relevant positions in the TCR β C-region. Differences between the human and murine TCR β C-regions were clustered in four domains. The influence of each domain on TCR expression was tested. Then, domains, which improved the expression in the murine variant were subdivided. Finally single amino acids were analyzed.

4.3.3 A domain of four amino acids within the TCR α chain supports improved expression of murinized TCR

The final construct of the NY-TCR β chain (β mm) was used to determine which amino acids of the murinized NY-TCR α chain contributed to improved TCR expression. We defined three areas for the C-region of the TCR α chain which covered all differences between the human and mouse sequences (Figure 24a). Then, we constructed three NY-TCR α chains with different C-regions, each containing one human and two murine domains (Figure 24b).

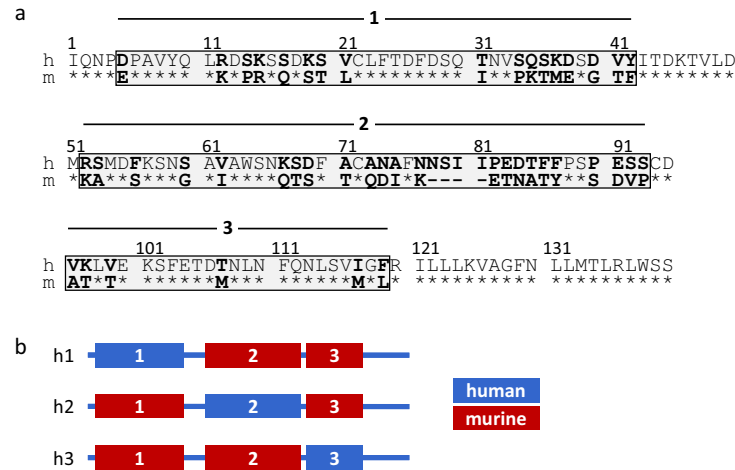


Figure 24: Comparison of human and murine TCR α C-regions. (a) The amino acid sequences of the human (h) and murine (m) TCR α C-region were compared and three domains covering all differences were defined (gray boxes). Asterisks represent the same amino acid in the murine sequence. (b) Schematic drawing of three different TCR α C-regions consisting each of one human and two murine domains.

The constructs were analyzed as described for the NY-TCR β chain and we identified domain 2 to be most critical for improved NY-TCR surface expression. Therefore, we cloned a construct containing only domain 2 from the murine sequence (α m2) (Figure 25a). Analogous to the strategy used for identification of critical amino acids in the C-region of the TCR β chain, domain 2 was then subdivided into smaller parts which were subsequently analyzed for NY-TCR expression.

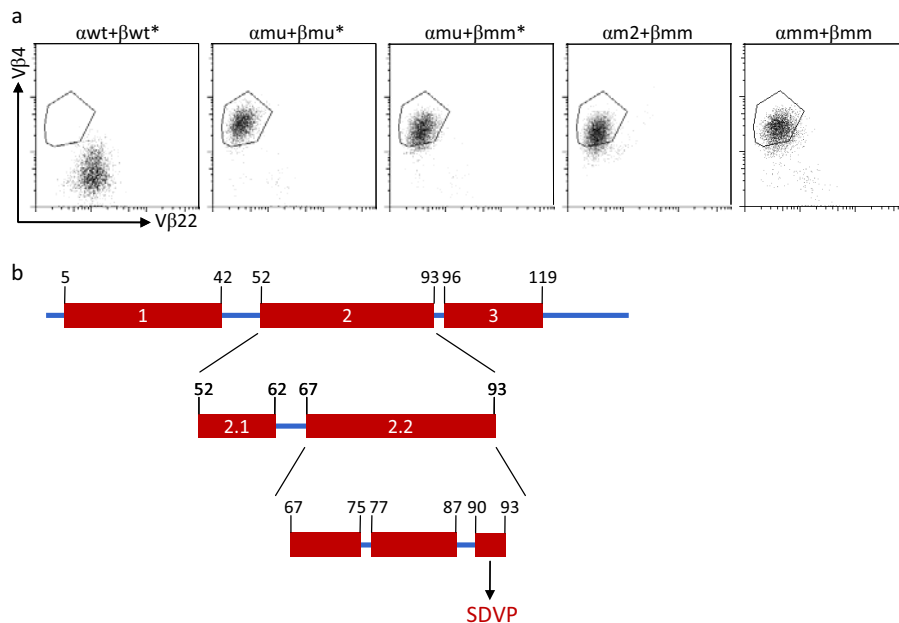


Figure 25: Enhanced TCR expression after murinization is maintained with only nine "murine" amino acids within the TCR C-regions. (a) J76/TCR26 cells were transduced with NY-TCR constructs containing different TCR α and TCR β C-regions and analyzed for surface expression of both TCR by staining with mAbs directed against V β 22 (TCR26) and V β 4 (NY-TCR). (b) Schematic summary of the procedures to identify the relevant positions in the TCR α C-region. *Pictures were taken from Figure 20a and Figure 22c for comparison.

Finally, we identified the region containing amino acids serine (S-90), aspartic acid (D-91), valine (V-92), and proline (P-93) as the most important segment. Single mutations of these amino acids did not reveal a preference for any single residue in this region. Thus, we elected to use the construct containing the four amino acids, S-D-V-P, of the murine sequence as the construct with the minimal essential TCR α modifications (α mm). The procedures used to identify the relevant positions in the TCR α C-region are summarized in Figure 25b. When compared to the combination of the completely murinized NY-TCR α chain (α mu) and the final NY-TCR β construct (β mm), the minimally murinized constructs (α mm+ β mm) led to similar NY-TCR expression levels and TCR26 replacement (Figure 25a, Table 5). Moreover, the provision of T cells with the final minimally murinized constructs of both the TCR α - and TCR β -chains (α mm+ β mm) led to TCR expression that was comparable to that of the completely murinized TCR (α mu+ β mu) (Figure 25a, Table 5).

In summary, we showed that the number of “murine” amino acids included in the sequence of the C-regions could be reduced by 90% (from 88 to 9), while retaining the improved surface expression of the modified TCR and replacement of the endogenous TCR.

4.3.4 Primary human T cells modified with minimally murinized NY-TCR show increased multimer binding and function compared to cells transduced with wild type TCR

Wild type, murinized, and minimally murinized TCR chains of NY-TCR were linked via the P2A element, to ensure simultaneous expression of both TCR chains, and transduced into J76 cells and human PBL. Pentamer-staining of transduced PBL revealed that the usage of murinized and minimally murinized NY-TCR chains enhanced the expression of NY-TCR compared to wild type chains (MFI of CD8/pentamer-positive cells: wt: 79, mu: 106, mm: 92) and percentage of positive cells (wt: 16, mu: 23, mm: 20) (Figure 26a). However, the improvement in expression was relatively low for both variants since wild type NY-TCR is already efficiently expressed on PBL (compare chapter 4.2.1). PBL were cocultured with NY-ESO-1 peptide pulsed T2 cells and supernatants collected 24 h later were analyzed for IFN- γ concentration. In agreement with data for surface expression, cells with the two murinized variants showed slightly increased IFN- γ concentrations (Figure 26b).

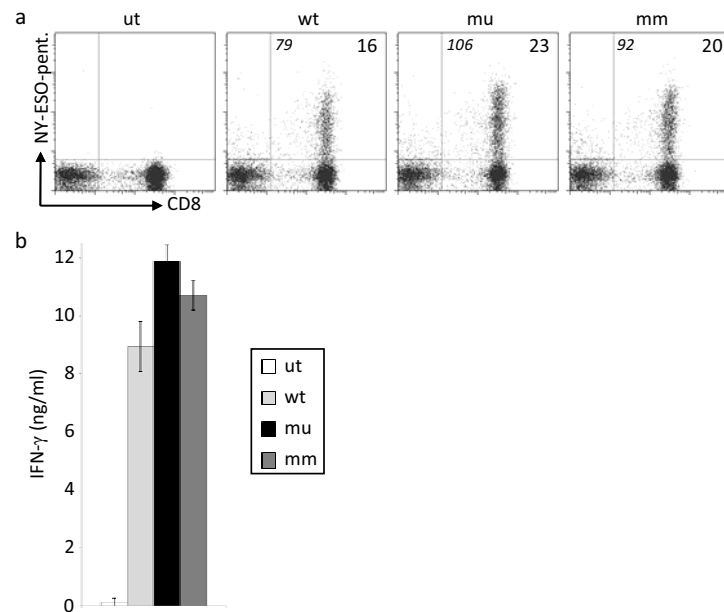


Figure 26: Enhanced expression of minimally murinized NY-TCR on human PBL. (a) PBMC were isolated from healthy donors and transduced with vectors containing wild type (wt), murinized (mu), or minimally murinized (mm) NY-TCR chains linked by a P2A element. Thirteen days after second transduction, PBL were stained with NY-ESO-pentamers and anti-CD8 mAb. Untransduced (ut) PBL were used as control. Numbers indicate MFI (italic) and percentages, respectively, of CD8/pentamer-positive cells. (b) PBL were cocultured with T2 cells pulsed with 1 μ M of NY-ESO-1 peptide and IFN- γ concentrations of different supernatants were analyzed by ELISA.

4.3.5 Primary human T cells modified with minimally murinized TCR T58 show increased multimer binding and enhanced tumor recognition compared to cells transduced with wild type TCR

To demonstrate that the identified amino acid exchanges which led to improved expression of NY-TCR are of general relevance we mutated the tyrosinase specific TCR T58 in the same way. Wild type, murinized and minimally murinized TCR genes were cloned as P2A vectors and transduced into human PBL. Ten days after transduction, TCR surface expression was analyzed by staining with tyrosinase-tetramers. Untransduced cells showed no tetramer binding (Figure 27a) and only a small portion of wt transduced cells bound the tyrosinase-tetramer (CD8/tetramer-positive: 1%, MFI: 72). Murinization improved the TCR expression (CD8/tetramer-positive: 9%, MFI: 116) and also minimal murinization resulted in enhanced tetramer binding compared to T58wt transduced cells (CD8/tetramer-positive: 5%, MFI: 92). Fifteen days after isolation, PBL were cocultured with melanoma cell lines Mel624.38 (tyrosinase⁺) and Mel-A375 (tyrosinase⁻) and collected supernatants were analyzed for IFN- γ concentration by ELISA. PBL transduced with T58mu released more (1172 pg/ml) IFN- γ than cells transduced with T58wt (311 pg/ml) (Figure 27b). In addition, PBL transduced with T58mm released more than the double amount (752 pg/ml) of IFN- γ compared to T58wt.

Untransduced cells did not react against Mel624.38 cells and the tyrosinase⁻ cell line Mel-A375 was not recognized by any of the PBL.

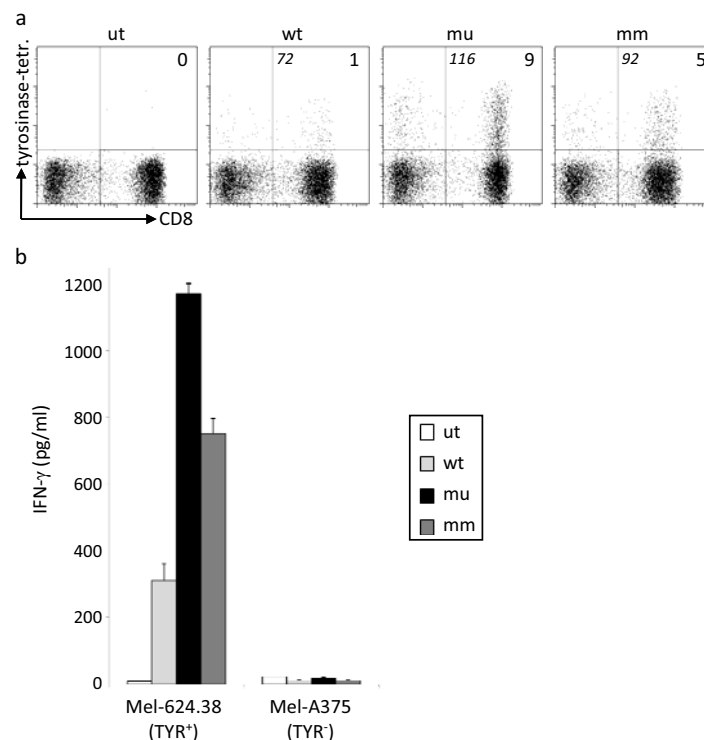


Figure 27: Minimal murinization improves the functional expression of the tyrosinase specific TCR T58. Primary human PBL were transduced with wild type (wt), murinized (mu), or minimally murinized (mm) TCR chains of the tyrosinase specific TCR T58. Cells were analyzed for cell surface expression thirteen days after transduction in comparison to untransduced cells (ut) by tyrosinase-tetramer and anti-CD8 staining. MFI (italic) and percentages of CD8/tetramer-positive cells are indicated. (b) Concentrations of IFN-γ after cocultivation with tyrosinase⁺ (Mel-624.38) and tyrosinase⁻ (Mel-A375) melanoma cell lines were analyzed by ELISA.

4.3.6 Primary human T cells modified with minimally murinized TCR53 show improved recognition of tumor cells compared to cells transduced with wild type TCR

As a third example we used the RCC reactive TCR53. Plasmids expressing wild type, murinized, and minimally murinized TCR53 chains in different combinations were constructed and transduced into J76 cells and human PBL. TCR expression, measured by anti-CD3 mAb staining of transduced J76 cells showed high TCR cell surface expression levels for all different combinations (Figure 28a) except for αwt/βmu, which even on cells lacking endogenous TCR could not be stably expressed, confirming the finding described in chapter 4.1.5, that trans-species mixed TCR are not expressed for all combinations of TCR chains. The transduced PBL were cocultured with RCC-26 cells and IFN-γ release into supernatant medium was measured by ELISA. PBL transduced with TCR53wt produced 2.1 ng/ml of IFN-γ, whereas PBL transduced with TCR53mu released substantially more IFN-γ (12 ng/ml) (Figure 28b). As expected, combinations of one TCR53wt chain and one TCR53mu chain did not yield

functional TCR expression and subsequently caused no release of IFN- γ above the background level. PBL transduced with TCR53mm produced more IFN- γ (9.5 ng/ml) compared to PBL transduced with TCR53wt and reached approximately 80% of those detected with cells expressing TCR53mu. Combinations of one TCR53mm and one TCR53wt chain resulted in low IFN- γ release comparable to TCR53wt, showing again that amino acid exchanges in both, TCR α and TCR β , C-regions were needed for enhanced functional expression of a TCR. PBL transduced with TCR53mm also released increased amounts of other cytokines such as TNF- α and IL-2, as compared to TCR53wt transduced cells (Figure 28b).

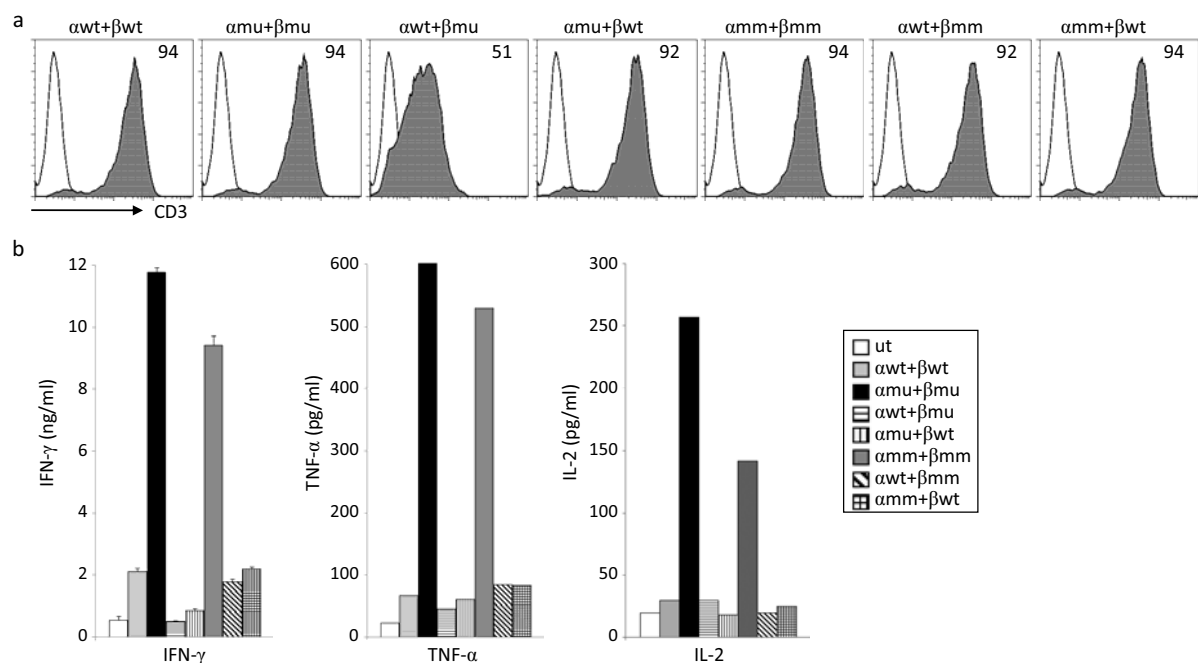


Figure 28: Minimal murinization of TCR53 enhances the tumor recognition of transduced human PBL. (a) J76 cells were transduced with combinations of wild type (wt), murinized (mu), and minimally murinized (mm) TCR chains of TCR53 and analyzed for TCR expression (CD3) four days after transduction (gray histograms) in comparison to untransduced cells (white histograms). Percentages of TCR-positive J76 cells are indicated. (b) PBMC were isolated from healthy donors and transduced in parallel to the J76 cells. Fifteen days after isolation, PBL were cocultured with the RCC cell line RCC-26 and concentrations of released cytokines were analyzed.

The analysis of TCR53 β (V β 20) expression after transduction of TCR53wt, TCR53mu, and TCR53mm into PBL revealed similar levels of TCR53 β expression on all cells (Figure 29). This demonstrated that minimal murinization, like complete murinization, enabled preferential pairing of the transferred TCR53 chains, as cells transduced with these constructs showed good function. In contrast, the TCR53wt chains seemed to pair mainly with endogenous TCR α chains to form mixed TCR heterodimers, as although the TCR53 β chain was expressed comparable to the other variants only little function was seen. This finding gives a hint that the improvement by murinization and also minimal murinization is due to preferential pairing of murinized TCR chains.

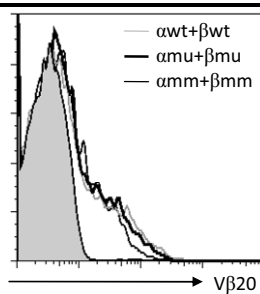


Figure 29: Preferential pairing of minimally murinized TCR. TCR53 transduced cells were stained with TCR53 β (V β 20) specific mAb in comparison to untransduced cells (filled gray histogram).

In summary, the previous examples indicated that minimal murinization not only led to an enhanced surface expression on primary human T cells, but resulted in an improved specific recognition of peptide pulsed cells and tumor cell lines.

5 Discussion

One precondition for a successful adoptive therapy with TCR gene modified T cells is a sufficient cell surface expression of the transferred therapeutic TCR. As T cells isolated from patients express endogenous TCR, theoretically cells with at least four different TCR combinations can arise, the endogenous, the therapeutic, and two mixed combinations. If a T cell with more than one endogenous TCR is transduced, even more different TCR α/β combinations may be expressed on the cell surface. This is possible as allelic exclusion is not complete, especially for the TCR α locus (Padovan *et al.*, 1993). Mixed TCR α/β combinations after TCR gene transfer into peripheral T cells have specificities, which are not screened in the thymus for recognition of self antigens. Therefore, these TCR α/β combinations bear the risk to induce autoimmunity, especially because of the high frequency of HLA alloreactive T cells in unselected T cell repertoires (Ford *et al.*, 1975). Furthermore, the expression of different TCR α/β combinations on one T cell dilutes the expression of the desired TCR, which may lead to non-reactive T cells.

5.1 The concept of strong and weak TCR

When the work for this thesis was started, it was not known which TCR α/β combinations are really expressed on the cell surface. Therefore, we created murine and human model systems to examine the mutual influence on TCR expression when different TCR are expressed simultaneously on one T cell. We transferred TCR α and TCR β chain genes into murine and human T cells. These cells expressed endogenous TCR with known specificities. Thus, in these models both, the endogenous and the transferred, TCR could be analyzed by Ab staining, MHC-multimer binding and functional assays. We found that different TCR are differently well expressed when they had to compete with a second TCR for surface expression. Based on these results, we developed the concept of strong and weak - in terms of surface expression - TCR. Some (strong) TCR were able to replace other (weak) TCR from the cell surface. Two TCR with similar strength were coexpressed. Our results were not completely unexpected, since it has been demonstrated that one TCR α chain paired better with a given TCR β chain than another TCR α chain (Couez *et al.*, 1991). Furthermore, in T cell hybridomas it had been shown that some TCR were expressed more efficiently than others (Saito *et al.*, 1989). A priori one might have thought that the TCR α/β heterodimer formation is primarily guided by the C-regions that are identical for any two TCR α/β combinations, with the exception of the two very similar TCR β C-regions. TCR α and TCR β chains are covalently linked through a disulfide bond between the C-regions and additionally, the C-regions are responsible for association with the invariant TCR chains. However, the preferential expression of certain TCR α/β combinations on the cell surface appears to be determined by the variable regions, e.g. the framework or the complementarity determining

regions of the TCR, as they include the only differences comparing two TCR. It will be important to determine the molecular mechanism(s) that distinguish strong and weak TCR. This might allow the reproducible transduction of T cells with strong TCR, resulting in exchange of antigen specificity and reducing the risk of unwanted side effects by the use of TCR gene modified T cells for adoptive immunotherapy. In the future TCR should not only be examined for their affinity towards the antigen, but in addition for their strength in matters of surface expression. Only the combination of both will guarantee an efficient TCR for therapy. The choice for an appropriate TCR will become easier if many TCR recognizing the preferred antigen are available and can be compared to each other.

At the moment we can only speculate about the reasons for the preferential expression of some TCR, e.g. it is currently not yet known whether it is regulated at the transcriptional level or post-transcriptionally. As in some experiments both TCR were expressed by the same promoter, it is unlikely that the level of expression differed significantly between both TCR, one of which was expressed on the cell surface, whereas the other was not. Therefore, it is more likely that TCR cell surface expression was regulated post-transcriptionally. A second possible reason might have been differences in mRNA stability due to motifs destabilizing the RNA, like cryptic splice sites. To exclude influences of unequal mRNA levels because of unequal transcription or RNA stability, identical amounts of mRNA should be confirmed, for example by real time PCR. The sequence of a TCR gene can have also an impact on the translation level as genes using codons coding for rare tRNA are insufficiently expressed. However, experiments comparing different codon-optimized TCR still showed differences in TCR expression levels arguing against differences of RNA stability and translation efficiency as the main cause for the observed phenomenon. Protein levels of different TCR chains could be analyzed by Western blot analysis or intracellular staining of TCR chains. However, unpaired TCR chains are degraded. Therefore, even inside the cells the concentration of a weak TCR would be clearly reduced. This is supported by intracellular staining of different TCR α/β combinations in $\gamma\delta$ T cells and the finding that the level of non-expressed TCR were also reduced intracellularly (Heemskerk *et al.*, 2007), however, this result has to be taken with caution, as it was not described whether binding to TCR molecules on the surface was blocked. Therefore, the staining may only reflect surface staining and not intracellular staining. In a second publication comparing surface and intracellular expression of TCR no differences in intracellular expression levels were described, although higher surface expression was found (Kuball *et al.*, 2007). However, intracellular staining intensity was very low for all TCR and, therefore, results are difficult to interpret.

If no transcriptional or translational influence plays a role, it is likely that the decision, whether a TCR α/β combination reaches the cell surface is made within the endoplasmatic reticulum and Golgi apparatus after translation. There, the different components of the TCR are assembled and chains,

which fail to assemble, are transported to the cytoplasm to be degraded. During assembly, different TCR α / β combinations have to compete for the invariant chains (CD3 and TCR ζ). It was shown that all TCR components apart from TCR ζ chains are produced in excess (Minami *et al.*, 1987). Therefore, the main reason for competition inside the endoplasmatic reticulum is the limited amount of TCR ζ chains. TCR α and TCR β chains, which can bind to each other more stably (strong TCR), are probably more often included into a correctly assembled TCR complex than weak TCR. Therefore, strong TCR will reach the cell surface, while weak TCR are degraded. Correctly assembled TCR complexes leaving the endoplasmatic reticulum and Golgi apparatus system seem to stay assembled also during endocytosis and recycling (von Essen *et al.*, 2004).

A further possibility to explain the enhanced surface expression of some TCR α / β combinations could be that weak TCR are present on the cell surface for only a short time due to a higher endocytosis or a lower recycling rate (Figure 4) compared to the second TCR. Furthermore, different half-life of different TCR could have an influence.

In addition, posttranslational mechanisms were suggested, which accomplishes clonality in cells expressing naturally two TCR α chains by suppressing one of them. In this process, TCR activation and protein tyrosine kinase Lck were involved (Niederberger *et al.*, 2003). Although this was not analyzed in detail, this mechanism seemed to play no role in our models. TCR activation may be more important during T cell development in the thymus and not for the *in vitro* generation of T cells.

To support the model that different affinities between different TCR α and TCR β chains are responsible for the preferred expression of some TCR, a direct measurement of the binding strength of TCR chains compared to their ability to be expressed would be interesting.

5.1.1 Exchange of antigen specificity

Analyzing the surface expression of different TCR, a complete TCR replacement could be shown for murine and human T cells. However, it could not be excluded that cells still expressed low amounts of the replaced TCR, which could not be detected with the methods used. Functional analysis revealed that in one situation (B3Z, Figure 11) the specificity of cells was completely exchanged as transduced cells no longer reacted to the peptide recognized by the replaced TCR. The advantage of this model was that transduced cells could be easily enriched to ~100% TCR expressing cells. This was unfortunately not possible for the situation when the human CTL line T21 was used, as these cells are difficult to culture, especially after transduction. Therefore, multiple steps of cell sorting were not possible. The remaining contamination with untransduced cells may be responsible for the recognition via the endogenous TCR. Approximately ten times more transduced cells were needed to reach the same specific lysis compared to untransduced cells. This would fit to the approximately 10% of cells which were either untransduced or could be stained with both tetramers.

Coexpression of two strong TCR led to reduced TCR expression levels of both TCR compared to cells expressing only one TCR. However, the function was not impaired, at least when using high peptide concentrations (Gladow *et al.*, 2004).

5.1.2 Mixed TCR on transduced T cells

We could verify the formation of mixed TCR on human cells, when transferring single TCR chains as we had shown before for murine T cells (Sommermeyer, 2004). Mixed TCR are most likely formed not only under this forced conditions but also in cells expressing both (TCR α and TCR β) transferred TCR chains, as after transduction of PBL often high percentages of cells expressing the transferred TCR β chain can be found although these cells do not bind tetramers or are hardly functional as shown for TCR53. The formation of mixed TCR after TCR gene transfer into a polyclonal population cannot be completely prevented if unmodified TCR are used, but the likelihood is reduced when TCR α and TCR β chains are used, which efficiently bind to each other.

Mixed TCR between human and murine chains are also possible but the formation is far less efficient. Therefore, the usage of murine TCR or parts of it (C-regions) reduces the formation of mixed TCR. Nevertheless it cannot be excluded that low amounts of mixed TCR are present on the cell surface.

5.1.3 Murine TCR are stronger than human TCR on human cells

The finding that murine TCR can replace human TCR on human cells is surprising, since J76 cells express human CD3 and TCR ζ components. One would expect that during evolution the TCR components from one species were optimized for their common expression. However, the expression of weaker TCR (like the human ones) might have also advantages, as the expression of less stable TCR may be regulated more easily. There are at least two possibilities, why murine TCR are more efficiently expressed on the cell surface, either the murine TCR α and/or TCR β chains assemble better with the invariant TCR chains or the two chains have a stronger binding to each other. Independent of the mechanism, which is responsible for the improved expression, the results suggest that mouse TCR or parts of them could be useful tools in a clinical setting.

5.2 TCR optimization

Great effort has been made to increase TCR protein levels and to enhance the preferential pairing and stability of transferred TCR α/β combinations (Uckert and Schumacher, 2009). We compared three promising methods of optimization either alone or in combination: (1) codon-optimization (Scholten *et al.*, 2006), (2) murinization (Cohen *et al.*, 2006), and (3) introduction of an additional interchain disulfide bond (Cohen *et al.*, 2007; Kuball *et al.*, 2007). All three methods had led to an increased surface expression of the modified TCR and an enhanced functional avidity of TCR

transduced T cells. Exchange of amino acids between the C-regions of TCR α and TCR β (Voss *et al.*, 2008) and hybrids with TCR ζ (Sebestyen *et al.*, 2008) were not used in the comparison as they showed no functional improvement in the results originally published. In addition the use of hybrids between TCR ζ and TCR α or TCR β chains creates new molecules, which may have altered properties compared to the original TCR. In a recent publication, it was shown that removing defined N-glycosylation sites in the TCR C-regions resulted in an enhanced functional avidity of transduced T cells (Kuball *et al.*, 2009). However, this strategy has not yet been compared to other strategies.

We found that there are differences in improvement when comparing the optimization strategies with different TCR. Not for all TCR all modifications had a great influence, e.g. murinization or additional disulfide bond only slightly increased the expression of NY-TCR. However, when using combinations of different strategies, all examined TCR could be clearly optimized. For some TCR, the optimization had a striking effect as cells transduced with unmodified TCR had only little or nearly no function and cells transduced with the modified TCR showed good function (TCR53 and IVS-B TCR). TCR with high expressions in their wild type version showed the smallest improvement after optimization (NY-TCR and D115 TCR). However, also for those TCR the optimization improved the expression of the TCR and the function of gene modified T cells.

Murinization was more efficient in our experiments than the introduction of an additional disulfide bond. However, we compared the modifications only with two TCR and for a final decision the evaluation of more TCR would be necessary. The combination of both strategies did not lead to further improvement in contrast to results published before (Cohen *et al.*, 2007). Codon-optimization seems to be helpful in any case, although also here the level of improvement depends on the TCR. Until now, no side effects due to codon-optimization were reported. A theoretical disadvantage might be the completely new nucleotide sequence, which can lead to new peptides when the sequence is translated with a frameshift. Frameshifts during translation can happen accidentally or are wanted, if two different proteins are translated from one common mRNA. The translation of new peptides might lead to immunogenicity.

The level of TCR surface expression seems to be defined by the combination of protein amount and strength of the TCR (affinity between TCR α and TCR β). Therefore, we suggest for the optimization of TCR a combination of codon-optimization and a modification leading to preferential pairing (like additional cysteine bond or murinization) (also supported by (Hart *et al.*, 2008)).

5.3 Minimally murinized TCR

Optimizing human TCR by exchanging the C-regions by their murine counterparts (murinization) was shown to be efficient in improving surface expression and to reduce the formation of mixed TCR

(chapter 4.2.2; (Cohen *et al.*, 2006; Hart *et al.*, 2008)). However, as the murine amino acid sequence is foreign to the human immune system, this strategy involves the risk of an unwanted immune reaction towards the therapeutic T cells.

Therefore, we identified the critical residues within the murine TCR α and TCR β C-regions, which are responsible for the improved functional expression of murinized TCR. As the overall structure of human and murine TCR is very similar and especially for the TCR β chain the sequence is homologous in many parts, it was possible to exchange small domains of the C-regions between human and murine TCR, to test which parts are responsible for the improved surface expression. In the TCR β C-region we identified one position (amino acid 18) to be essential, in which the human sequence contains an acidic (glutamic acid) and the murine sequence a basic (lysine) amino acid. This exchange from a negatively charged side chain to a positively charged one seems to confer a large difference in stability of the TCR complex. This stabilizing effect was not restricted to lysine, but could also be achieved with arginine as a further basic amino acid. Interestingly, mice seem to be exceptional when compared to other mammals, since they have either lysine (*Mus musculus*) or arginine (*Mus spretus*) at this position, whereas other mammals for which TCR sequences are available (chimpanzee, rhesus monkey, rat, rabbit, dog, cattle, sheep, and pig), have glutamic acid at this position (Figure 30). In addition, we found four amino acids (A-22, I-133, A-136, and H-139) in the TCR β C-region that further increased the stability of the TCR. For the TCR α chain, we identified a segment of four amino acids (S-90, D-91, V-92, and P-93), which is mainly responsible for the enhanced expression of murinized TCR.

Mus musculus	PSKAEI
Mus spretus	PSRAEI
human	PSEAEI
chimpanzee	PSEAEI
rhesus monkey	PSEAEI
dog	PSEAEI
cattle	PSEAEI
sheep	PSEAEI
pig	PSEAEI
rat	PSEAEI
rabbit	PSEAEI

Figure 30: Alignment of amino acids 16-21 of the TCR β C-region from different mammals.

We used the structure of the mouse 2C-TCR (Garcia *et al.*, 1996) as a model to localize the positions of the various relevant residues that we identified within the TCR structure. Interestingly, these amino acids seem to be located in the same area of the TCR. In the structure of the 2C-TCR, they are at the base of the crystallized structure at the transition from the immunoglobulin domains to the connecting peptide (Figure 31). For the TCR β chain, only the two amino acids K-18 and A-22 are included in the crystallized region of the 2C-TCR; the remaining three amino acids of part 4.1 (Figure

22 e) directly follow the crystallized region (orange arrow, Figure 31) and are, therefore, perhaps also located in the same area as the other important amino acids we identified. When analyzing the structure, it seems plausible that the basic lysine K-18 of the TCR β chain and the acidic aspartic acid D-91 of the TCR α chain interact with each other. In line with this hypothesis, a mutation of aspartic acid D-91 to a lysine led to a reduced TCR surface expression, but it cannot be excluded that this mutation destroyed the secondary structure of this region. However, since the human sequence also has an acidic amino acid at position 91 of the TCR α chain (E-91) there is no obvious explanation why it is important for this region to be of murine origin. One could speculate that an altered distribution of inflexible proline residues contributes to the effect, since two prolines at positions 88 and 90 are directly N-terminal to the glutamic acid E-91 in the human sequence. The resultant greater flexibility might facilitate an interaction of the positively charged TCR β chain with the negatively charged TCR α chain. However, structural data of the part, where the decisive amino acids in the TCR α chain are located have to be taken with caution, because this part lies at the very end of the crystallized structure and is therefore not accurate.

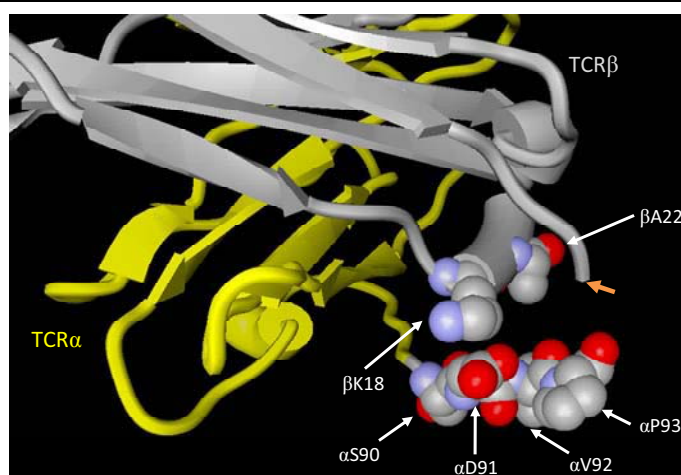


Figure 31: Analysis of the 2C-TCR structure reveals proximity of the identified critical amino acids. View on the bottom of the crystallized part of the 2C-TCR (Protein Data Bank, accession number: 1TCR) (Garcia *et al.*, 1996) shows the identified amino acids of the TCR α (yellow) and TCR β (gray) chains. Critical amino acid residues are shown in detail and are labeled. The orange arrow marks the C-terminus of the crystallized part of TCR β , where the identified amino acids of domain 4.1 would follow.

Extracellular binding to CD3 components of the TCR are difficult to predict, although there are models for CD3 binding (Kuhns *et al.*, 2006; Kuhns and Davis, 2007). However, there is no crystal structure of the whole TCR complex and therefore, a coherent model is still missing. Because the minimally murinized variants possess the same transmembrane region as wild type human TCR, there should be no differences in the assembly with the invariant TCR chains within the

transmembrane regions. In addition, the important amino acids for transmembrane contact are conserved across many species.

The usage of the minimally murinized TCR C-regions improved not only the expression of the tested TCR compared to wild type TCR but also the function of transduced PBL. However, exchange of only nine amino acids did not completely achieve the levels of expression of completely murinized C-regions. Dependent on the specific TCR, the minimally murinized variants yielded between 50% and 85% of TCR expression and function when compared with fully murinized TCR. To achieve effects closer to 100%, it is likely that additional (less prominent) amino acids must also be exchanged. However, when considering clinical application it is important to balance a somewhat better TCR surface expression against increased risk of immunogenicity due to the insertion of more foreign residues. When considering this point, one could think about further reducing the number of “murine” amino acids within the TCR β C-region to the single most important lysine at position 18.

The FG-loop of the murine TCR β C-region is a known epitope for antibodies (Ghendler et al., 1998), and therefore, most likely also immunogenic in humans. In our minimally murinized TCR β variant, this loop (included in domain β 3) was completely “re-humanized” and consequently, the probable immunogenicity of this region was avoided.

In our experiments we used the murine TCRC β 2-region for substitution since the NY-TCR and TCR53 utilize a human TCRC β 2-region. However, the identified minimally murinized TCRC β 2-region could also be employed to equip human TCR utilizing a TCRC β 1-region. The tyrosinase specific TCR T58, which contains a TCRC β 1-region, also showed enhanced functionality with the minimally murinized C-regions.

PBL transduced with minimally murinized TCR functioned well *in vitro*, and cells revealed no abnormalities like reduced growth rates or increased numbers of dead cells. However, one cannot exclude that the new C-regions have altered functions *in vivo*, as already small changes in the sequence can have a large impact; e.g., it has been shown in a mouse model, that a point mutation in the TCR β transmembrane domain impaired the development and function of CD8⁺ memory T cells (Teixeiro *et al.*, 2009). Therefore, a further extensive functional characterization of cells transduced with minimally murinized TCR is needed before they can be used in a clinical setting.

In conclusion, we have identified a set of amino acids in murine TCR C-regions which - if used for the replacement of the corresponding counterparts in human TCR - led to an improved TCR cell surface expression and an increased functional avidity of TCR gene modified T cells. These results have important implications on the design of TCR selected for use in TCR gene therapy.

5.4 Additional options to ensure high surface expression of therapeutic TCR

A possibility to ensure high expression levels of only the therapeutic TCR without the interference of additional (endogenous) TCR is the usage of hematopoietic stem cells for transduction, as the rearrangement of the TCR loci is inhibited in most cells due to the transduced TCR chain genes. However, transduction of stem cells bears the increased risk of insertional mutagenesis as seen after common γ chain gene therapy of SCID-X1 patients (Hacein-Bey-Abina *et al.*, 2003). The risk is reduced for TCR gene transfer compared to the common γ chain, as high expression of the common γ chain gives a clear growth advantages. A second problem of stem cells is, that T cells developing from stem cells undergo selection in the thymus, which could lead to the deletion of cells, when tumor associated antigens are used as a target.

Transfer of TCR α/β chain genes into $\gamma\delta$ T cells was also proposed to avoid mixed TCR, as TCR α/β chains cannot pair with TCR γ/δ chains (van der Veken *et al.*, 2006). But, functional *in vivo* data with these cells were not convincing as in mice expressing high antigen levels in the pancreas no signs of autoimmunity were reported (van der Veken *et al.*, 2009). This was not unexpected because $\gamma\delta$ T cells are thought to be involved in the innate immune system (Casetti and Martino, 2008) and, therefore, it is unlikely that $\gamma\delta$ T cells are equally functional as $\alpha\beta$ T cells when transduced with TCR α/β chains.

A further option is the downregulation of endogenous TCR in transduced cells. This might be possible using RNA interference (RNAi) technologies (Meister and Tuschl, 2004) by transferring short hairpin RNA or micro RNA directed against the C-regions of TCR α and TCR β together with the TCR genes into the cells. However, the downregulation of TCR genes is not trivial as TCR α and TCR β chains are synthesized in excess (Minami *et al.*, 1987) and therefore, even if most of TCR mRNA is downregulated, the surface expression might stay unaffected. Using new efficient systems for knock down of endogenous TCR, it might be possible in the future to reach a sufficient downregulation. This technique might make the optimization of therapeutic TCR redundant. The complete downregulation of the endogenous TCR would create the situation of empty T cells like J76 cells where the expression of even weak TCR should not be problematic. Only the nucleotide sequences of the therapeutic TCR would have to be slightly changed, so that they are not recognized by the RNA interference system.

5.5 TCR gene therapy

The efforts of many laboratories including the work presented in this thesis have led to the situation, where therapeutic TCR can be provided that should work in a clinical setting even if a weak TCR is planned to be used. As shown for TCR53, which did hardly function in its wild type form, these TCR can be optimized to well functioning TCR. Of course, further improvements are possible, especially

considering the specific downregulation of endogenous TCR by RNA interference or other mechanisms.

On the vector side, nowadays good γ -retroviral vectors, like MP71, can be used for the efficient transduction of T cells also under “good manufacturing practice” (GMP) conditions, which are required if cells are used in the clinic. To further improve the vector system also lentiviral vectors were developed (Bobisse *et al.*, 2007). They have the advantage, that also non-dividing cells can be transduced. In addition, further improvement of the viral envelope proteins, which are responsible for the infected cell type, is possible. For example, the usage of modified envelop proteins of the measles virus to pseudotype lentivirus created the option of targeting only specific cell populations (Funke *et al.*, 2008). The usage of T cell specific envelopes would have the advantage that only T cells and no other cell types are transduced when a cell mixture like PBMC are incubated with viral supernatant. Also non-viral vector systems, for example based on transposons can be further improved for TCR gene transfer (Peng *et al.*, 2009). The ideal transfer system would include the specific exchange of the endogenous TCR genes by the transferred ones by homologous recombination. This would on the one hand eliminate the risk of insertional mutagenesis and on the other hand leads to a complete exchange of expressed TCR and thereby an exchange of antigen specificity. Furthermore, the risk of mixed TCR and the activation of tolerant T cells would be avoided and a high expression level of the therapeutic TCR would be ensured due to the absence of competition.

A third decisive factor to avoid the failure of TCR gene modified T cells is the activation status of cells and the time span of cultivation (Gattinoni *et al.*, 2006). Cultivation of T cells *in vitro* leads to their differentiation. These cells often have increased anti tumor activity *in vitro*, but are less functional *in vivo*. Therefore, it seems to be important that T cells are transferred back into the patient as soon as possible. In addition, it is advantageous if cells are only gently activated. However, with short culture periods and mild activation it will not be possible to reach the high cell numbers, which are used at the moment. Yet, these cells should have a superior function which can compensate for the lower number. It was shown in a mouse model that even a single naive CD8⁺ T cell precursor can develop into diverse effector and memory subsets (Stemberger *et al.*, 2007). In addition, the transfer of the right subset of cells seems to be decisive for a persistent function of transferred T cells *in vivo* (Berger *et al.*, 2008).

The most important issue for a successful application of TCR gene therapy is the choice of the right antigen used as a target. Clinical studies using the tumor associated antigens Melan-A and gp100 showed low response rates (30% and 19%, respectively) but a high incidence of autoimmunity (80% and 94%, respectively) (Johnson *et al.*, 2009). In these studies normal cells expressing the antigen were apparently better recognized than tumor cells, even though the antigen level might have been

higher on tumor cells. This was not unexpected as tumors are well protected against hits from the immune system (Schreiber, 2003).

The usage of tumor specific antigens will avoid the destruction of normal tissue and will make the therapy safer. More importantly, it is very likely that targeting tumor specific antigens will improve the success rate of TCR gene therapy. The usage of tumor associated antigens is attractive as many patients can be treated with the same TCR. But even if the therapy with tumor specific TCR has to be individualized for many patients and will therefore be very expensive, it will establish if it is more successful.



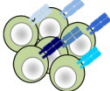

		Status	Outlook
	TCR	Optimization strategies like murinization, additional disulfide bond and codon-optimization enable high TCR expression levels.	Knock-down of endogenous TCR would further enhance expression of therapeutic TCR and would minimize the risk of mixed TCR.
	Vector	Efficient gene transfer with γ -retroviral and lentiviral vectors is possible.	Exchange of endogenous TCR genes by homologous recombination would be ideal.
	Phenotype of cells	Short period of <i>in vitro</i> culture and moderate cell activation are advantageous.	Further characterization, which T cell subsets are best will be needed.
	Targeted antigen	Tumor associated antigens are targeted, which are also expressed on normal cells.	Tumor specific antigens should be targeted to reduce side effects and to enhance the success rate of T cell therapy.

Figure 32: Summary of decisive factors for TCR gene therapy.

Abbreviations

Ab	antibody
APC	allophycocyanin
C	constant
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDR	complementarity determining region
CMV	cytomegalovirus
co	codon-optimized
CTL	cytotoxic T lymphocyte
cys	with additional cystein bond
cysco	with additional cystein bond and codon-optimized
D	diversity
D-MEM	Dulbecco's modified Eagle's medium
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanat
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
J	joining
J76	Jurkat76
LTR	long terminal repeat
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mm	minimally murinized
mu	murinized
muco	murinized and codon-optimized
mucys	murinized and with additional cystein bond

OVA	ovalbumin
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PRE	posttranscriptional regulatory element
RCC	renal cell carcinoma
TCR	T cell receptor
TIL	tumor infiltrating lymphocytes
V	variable
wt	wild type

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Statement

Hiermit erkläre ich gemäß der Promotionsordnung der Humboldt-Universität zu Berlin vom 01. Oktober 2002, dass ich

- die vorliegende Arbeit eigenständig unter Anleitung und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.
- die Arbeit in gleicher oder ähnlicher Form nicht in anderen Promotionsverfahren vorgelegt wurde.
- mir die geltende Promotionsordnung vom 01. Oktober 2002 bekannt ist.

Berlin, den 28.07.2009

Unterschrift